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TITLE: Pharmacoligcal and Behavioral Enhancement of Neuroplasticity in the MPTP-Lesioned Mouse and Non-Human Primate

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Introduction

The primary focus of this research proposal is to determine the underlying mechanisms responsible for neuroplasticity in the injured adult basal ganglia. For these studies we utilize the neurotoxicant MPTP that selectively destroys nigrostriatal dopaminergic neurons and leads to the depletion of striatal dopamine as well as the development of parkinsonian features. In the squirrel monkey these features include slowness of movement, balance impairment and diminished hand dexterity. In our laboratory we utilize both the MPTP-lesioned C57BL6 mouse and the MPTP-lesioned squirrel monkey. Both models show intrinsic plasticity through either striatal dopamine return (mouse) and/or behavioral recovery (squirrel monkey). In this proposal we were particularly interested in understanding whether exercise (mouse) or dopamine replacement therapy (monkey) might enhance intrinsic neuroplasticity of the injured basal ganglia. For this purpose, the proposal was divided into two components, a mouse exercise study and a squirrel monkey dopamine replacement study. These studies were designed to be complementary in that both nonpharmacological and pharmacological effects of neuroplasticity are being investigated.

In the following sections are included the abstract, introduction and specific aims from the original proposal. This is followed by the accomplishments and research outcomes from year one. This annual report also includes manuscripts in the form of appendices.

Abstract (From the Original Application)

The purpose of this proposal is to investigate the molecular mechanisms involving pharmacological and behavioral (exercise) enhanced neuroplasticity of the injured basal ganglia. Our central hypothesis is that exercise and pharmacological intervention, specifically the administration of a D2 dopamine-receptor agonist, enhances neuroplasticity by modulating glutamate-dopamine interactions. The following proposal has two complementary components using two animal models to address the molecular mechanisms underlying exercise- and pharmacologically-enhanced neuroplasticity. Using the MPTP C57BL/6 mouse **Component One** will test the hypothesis that exercise enhances plasticity of the MPTP-injured basal ganglia through glutamate by modulating dopamine biosynthesis. This hypothesis will be tested through changes in dopamine, and proteins involved in dopamine biosynthesis and uptake (tyrosine hydroxylase and dopamine transporter) and changes in glutamatergic synapses and receptor subtype. This hypothesis will be further tested through determining whether exercise-enhanced neuroplasticity may be attenuated with a glutamate antagonist. Using the MPTP-lesioned non-human primate **Component Two** will test the hypothesis that the administration of a D2 receptor agonist (Pramipexole) enhances neuroplasticity of the MPTP-injured basal ganglia through its effect on pre- and post- synaptic dopamine biosynthesis, uptake and receptor expression as well as glutamatergic synapses. This hypothesis will be tested through changes in dopamine and its metabolites, proteins involved in dopamine biosynthesis, uptake, and storage (tyrosine hydroxylase, dopamine transporter, and vesicular monoamine transporter), changes in dopamine receptor subtypes and their respective neuropeptides, and changes in glutamatergic synapses. By elucidating the role of exercise and pharmacological manipulation in neuroplasticity of the injured brain we hope to identify novel therapeutic targets for the treatment of brain injury and neurotoxic insult. Since military personnel are at risk for a wide range of brain injuries including head trauma, neurotoxic exposure (from pesticides, hostile enemy poisoning, viral and biological weapon based agents) it is imperative that medical strategies be made available to reverse the debilitating neurological deficits.

D: STATEMENT OF WORK
From the original Application

The brain's capacity for recovery from damage is far greater than previously recognized. It is now understood that neuroplasticity can be modulated through activity-dependent processes including exercise and environmental enrichment, and through pharmacological manipulation. Most of our understanding of exercise and pharmacological enhanced neuroplasticity is derived from studies in the cortex and the hippocampus, but there is mounting evidence that the same phenomenon occurs in the injured basal ganglia. The molecular mechanisms for this phenomenon are not well understood. Using two animal models of injury induced neuroplasticity in the basal ganglia (the MPTP-lesioned mouse and MPTP-lesioned non-human primate) we propose to examine two modes of intervention to enhance neuroplasticity. These include exercise in the MPTP-lesioned mouse model and pharmacological intervention in the MPTP-lesioned non-human primate. Our central hypothesis is that exercise and pharmacological intervention, specifically the administration of a D2 dopamine-receptor agonist, enhances neuroplasticity by modulating glutamate-dopamine interactions.

The following proposal has two complementary components using both animal models to address the molecular mechanisms underlying exercise- and pharmacologically-enhanced neuroplasticity. Using the MPTP C57BL/6 mouse **Component One** will test the hypothesis that exercise enhances plasticity of the MPTP-injured basal ganglia through glutamate by modulating dopamine biosynthesis. This hypothesis will be tested through changes in dopamine, and proteins involved in dopamine biosynthesis and uptake (tyrosine hydroxylase and dopamine transporter) and changes in glutamatergic synapses and receptor subtype. This hypothesis will be further tested through determining whether exercise-enhanced neuroplasticity may be attenuated with a glutamate antagonist. Using the MPTP-lesioned non-human primate **Component Two** will test the hypothesis that the administration of a D2 receptor agonist (pramipexole) enhances neuroplasticity of the MPTP-injured basal ganglia through its effect on pre- and post- synaptic dopamine biosynthesis, uptake and receptor expression as well as glutamatergic synapses. This hypothesis will be tested through changes in dopamine and its metabolites, proteins involved in dopamine biosynthesis, uptake, and storage (tyrosine hydroxylase, dopamine transporter, and vesicular monoamine transporter), changes in dopamine receptor subtypes and their respective neuropeptides, and changes in glutamatergic synapses. By elucidating the role of exercise and pharmacological manipulation in neuroplasticity of the injured brain we will identify new therapeutic targets for the treatment of traumatic brain injury and neurotoxic insult, two high-risk morbidities that are common to military personnel.

<u>Component One</u>: To test the hypothesis that exercise enhances neuroplasticity of the MPTP-lesioned mouse through glutamate by modulating dopamine biosynthesis.

Component One will utilize the following 4 treatment groups for **Study1** through **Study 4**:

(1) Saline-injected;
(2) MPTP-injected;
(3) Saline-injected + exercise;
(4) MPTP-injected + exercise.

Study 5 will utilize the following glutamate antagonists: AMPA antagonist (GYKI-52466) and the NMDA antagonist (MK-801) in the following 8 treatment groups:

(1) Saline-injected + GYKI-52466;
(2) MPTP-injected + GYKI-52466;
(5) Saline-injected + MK801;
(6) MPTP-injected + MK801;

(3) Saline-injected + exercise + GYKI-52466;
(4) MPTP-injected + exercise + GYKI-52466;
(7) Saline-injected + exercise + MK801;
(8) MPTP-injected + exercise +MK801.

Exercise will be performed on a motorized rodent treadmill. Brain tissue will be collected after 30 days of running.

Study 1: The level of striatal dopamine and its metabolites will be determined using HPLC analysis comparing exercise versus non-exercise groups in the MPTP-lesioned mouse.

Study 2: The pattern of expression of striatal tyrosine hydroxylase (TH), dopamine transporter (DAT), cAMP-responsive enhancer binding protein (CREB), phospho~CREB, and dopamine- and adenosine-3':5'-monophosphate-regulated phosphoprotein (DARPP-32), and phospho~DARPP-32 protein and their mRNA transcripts in surviving dopaminergic neurons will be determined using immunohistochemistry, western immunoblotting, *in situ* hybridization and correlated with striatal dopamine return. Pilot data shows attenuation of the return of DAT protein, and TH mRNA by exercise in MPTP-lesioned mice.

Study 3: The effect of exercise on glutamatergic synapses in the striatum after injury will be determined using ultrastructural immunohistochemical staining with electron microscopy. Pilot data shows altered glutamatergic synapses using immuno-electron microscopy.

Study 4: The pattern of expression of subunits for both the NMDA and AMPA receptor subtypes and their phosphorylated state will be determined using western immunoblotting, immunocytochemistry and *in situ* hybridization histochemistry.

Study 5: *We will test the hypothesis that exercise induced neuroplasticity can be attenuated through the administration of either a NMDA or AMPA receptor antagonist*. After MPTP-lesioning mice will be subjected to exercise while receiving either the NMDA receptor antagonist MK-801 or the AMPA receptor antagonist GYKI-52466. Behavioral recovery will be compared between groups. Brain tissue will be analyzed for alteration in dopaminergic function (dopamine, DAT and TH expression). Pilot studies show that both glutamate receptor antagonists GYKI-52466 and MK-801 can be administered in this model of MPTP-lesioning.

Component Two: To test the hypothesis that the administration of a D2 receptor agonist (pramipexole) enhances neuroplasticity of the MPTP-lesioned non-human primate through its effect on dopamine (biosynthesis, uptake, and receptor expression) and glutamatergic synapses.

Component Two will utilize the following treatment groups (n = 4 per group):
(1) Saline-injected harvested at 6 weeks after the last injection;
(2) Saline-injected harvested at 16 weeks after the last injection;
(3) MPTP-injected harvested at 6 weeks after the last injection;
(4) MPTP-injected harvested at 16 weeks after the last injection;
(5) Saline-injected + pramipexole harvested at 6 weeks after the last injection;
(6) Saline-injected + pramipexole harvested at 16 weeks after the last injection;
(7) MPTP-injected + pramipexole harvested at 6 weeks after the last injection;
(8) MPTP-injected + pramipexole harvested at 16 weeks after the last injection.

Study 1: The behavioral recovery of saline injected and MPTP-lesioned squirrel monkeys will be compared with and without the administration of pramipexole. Animal behavior will be monitored using both a cage side clinical rating scale and a personal activity monitor.

Study 2: The pattern of expression of proteins and mRNA transcripts important for dopaminergic function, (including TH, DAT, VMAT2) at the level of the SNpc and CPu will be determined. Preliminary data supports our ability to carry out western immunoblotting, immunocytochemistry and *in situ* hybridization in the MPTP-lesioned non-human primate.

Study 3: The pattern of expression of the dopamine receptors D1, D2, and D3 will be determined in both the SNpc and CPu. The level of protein expression will be determined western immunoblotting, immunohistochemistry, while the level of mRNA transcript expression will be determined using *in situ* hybridization histochemistry. Double labeling techniques will be used to co-localize the dopamine receptor changes with other enkephalin or substance P containing neurons. Preliminary data supports our ability to use these techniques in the non-human primate.

Study 4: The effect of pramipexole on glutamatergic synapses in the striatum after injury will be determined using ultrastructural immunohistochemical staining with electron microscopy. Pilot data shows our ability to quantify glutamatergic synapses using immuno-electron microscopy.

At the conclusion of these studies we will have a better understanding on the role of exercise and dopamine agonist (pramipexole) treatment in enhancing neuroplasticity of the injured basal ganglia in the mouse and the non-human primate. This may then identify important therapeutic targets (through glutamate and dopamine) for the treatment of brain injury.

Table 1: Timeline of Experimental Design for Component One (Exercise in the MPTP-Lesioned Mouse Model).

Component One: Exercise in the MPTP-lesioned Mouse				
	Year 1	**Year 2**	**Year 3**	**Year 4**
Study 1: Analysis of Dopamine and its metabolites	HPLC			
Study 2: Analysis of TH, DAT, CREB, and DARPP-32	Immunocytochemistry, In Situ Hybridization, Western Immunoblotting			
Study 3: Analysis of striatal glutamate synapses		Immuno-electron microscopy		
Study 4: Analysis of NMDA and AMPA receptor subtypes		Immunocytochemistry, In Situ Hybridization, Western Immunoblotting		
Study 5: Attenuate neuroplasticity with NMDA and AMPA receptor antagonists			Immunocytochemistry, In Situ Hybridization, Western Immunoblotting, Immuno-electron microscopy	

<u>Component 2: Pharmacological Enhancement of Neuroplasticity in the MPTP-lesioned Non-Human Primate Model.</u>

Time Line:

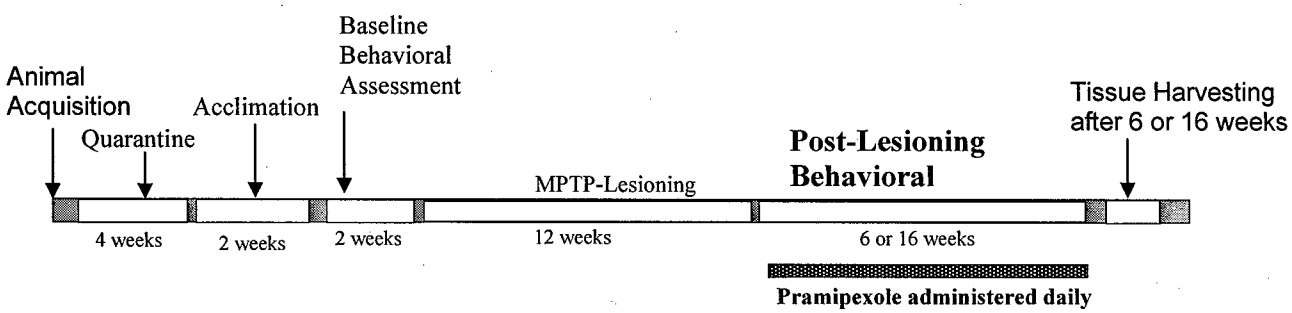


Table 2:

Specific Aim	Year 1	Year 2	Year 3	Year 4
	Lesion animals and administer Pramipexole			
Study 1	Behavioral analysis			
Study 2		TH, DAT, VMAT mRNA and protein using WIB, ICC and ISH		
Study 3		Dopamine Receptor D1, D2, and D3 using WIB, ICC, and ISH		
Study 4		Analysis of glutamatergic synapses using immuno-EM		

Key Research Accomplishments for Year One

Component One: Enhancement of neuroplasticity in the MPTP-lesioned mouse

(i) Intensive treadmill exercise does not increase the level of striatal dopamine in the MPTP-lesioned mouse model of basal ganglia injury.

(ii) Intensive treadmill exercise suppresses the intrinsic return of striatal tyrosine hydroxylase and dopamine transporter proteins in the MPTP-lesioned mouse.

(iii) Intensive treadmill exercise suppresses the expression of tyrosine hydroxylase and dopamine transporter mRNA transcripts in both saline + exercise and MPTP + exercise mice.

(iv) Intensive treadmill exercise causes a normalization of synaptic glutamate to levels seen in non-lesioned mice without exercise.

(v) The administration of AMPA and NMDA receptor antagonists altered the pattern of expression of tyrosine hydroxylase and dopamine transporter mRNA transcription in nigrostriatal dopaminergic neurons as well as the pattern of expression of striatal tyrosine hydroxylase.

Component Two: Enhancement of neuroplasticity in the MPTP-lesioned nonhuman primate.

(i) The administration of the dopamine agonist pramipexole induces dyskinesia.This occurs later then that seen with sinemet administration.

(ii) There is no detectable enhancement of intrinsic behavioral recovery within the first 6 weeks after MPTP-lesioning with either sinemet or pramipexole.

(iii) Pramipexole slightly increases dopamine levels in both the MPTP-lesioned mouse and MPTP-lesioned squirrel monkey.

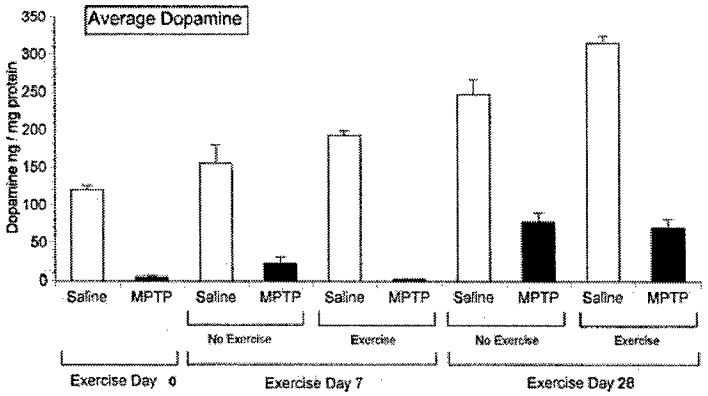
Reportable Outcomes

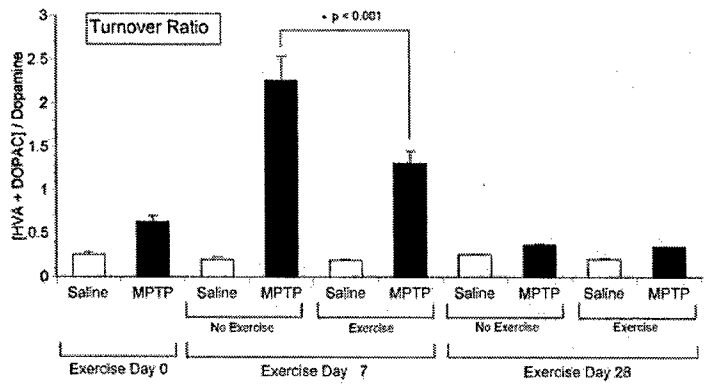
Component 1: To test the hypothesis that exercise enhances neuroplasticity of the MPTP-lesioned mouse through glutamate by modulating dopamine biosynthesis.

***Study 1**: The level of striatal dopamine and its metabolites will be determined using HPLC analysis comparing exercise versus non-exercise groups in the MPTP-lesioned mouse.*

This Aim has been completed in Year 1. A manuscript describing these studies is in preparation for publication. A copy of this manuscript is included as Appendix 2 in this Report.

Briefly, an important question to be addressed in this proposal is the effect of intensive treadmill exercise in the MPTP-lesioned mouse model of basal ganglia injury on the level of striatal dopamine and its metabolites. Mice were lesioned with MPTP in a series of 4 injections at a concentration of 20 mg/kg (free-base) while another group of mice were administered saline. Mice from both the MPTP-lesioned and saline groups were subjected to either intensive treadmill exercise (1 hour per day) or no exercise for 28 days. During the exercise paradigm brain tissues were harvested at either 7 or 28 days of exercise. Striatal tissues were analyzed for dopamine and its metabolites (HVA and DOPAC) as well as the pattern of expression of protein for tyrosine hydroxylase (TH) and the dopamine transporter (DAT). Since our previous manuscript published in Year 1 of this proposal (attached as Appendix 2 of this proposal) showed that the return of DAT protein expression in the striatum is actually suppressed through intensive treadmill exercise. Another manuscript published in Year 1 of this proposal (and included as Appendix 1 in this Report) showed that there is intrinsic return of both TH and DAT protein expression 2 to 3 months after MPTP-lesioning. Initially, we expected that intensive treadmill exercise would enhance the return of these markers of basal ganglia intensity since it enhanced (accelerated) behavioral recovery (based on treadmill running duration and velocity). The suppression of DAT protein expression was in fact an unexpected outcome that we continue to pursue. Since this outcome acted as an indicator of the effect of our intensive treadmill exercise paradigm we now routinely use it to validate our intervention. The outcome of this study is that the intensive treadmill exercise paradigm does not accelerate the return of striatal dopamine. Measurement at both 7 and 28 days of exercise showed levels of striatal dopamine not to be significantly different from MPTP-lesioned non-exercise animals. Therefore, these results indicate that the enhancement of behavioral recovery in the MPTP-lesioned mouse is not apparently due to the return of striatal dopamine. Analysis of striatal glutamate using immuno-electron microscopy showed elevation in glutamate indicating that another neurotransmitter system (in this case originating from the cortico-striatal pathway) plays an important role in behavioral recovery. These results are described in a manuscript (Fisher et al 2004, Appendix 2).





There are no deviations from Study 1.

***Study 2**: The pattern of expression of striatal tyrosine hydroxylase (TH), dopamine transporter (DAT), cAMP-responsive enhancer binding protein (CREB), phospho~CREB, and dopamine- and adenosine-3':5'-monophosphate-regulated phosphoprotein (DARPP-32), and phospho~DARPP-32 protein and their mRNA transcripts in surviving dopaminergic neurons will be determined using immunohistochemistry, western immunoblotting, in situ hybridization and correlated with striatal dopamine return. Pilot data shows attenuation of the return of DAT protein, and TH mRNA by exercise in MPTP-lesioned mice.*

As mentioned in Study 1, both striatal TH and DAT protein return after MPTP-lesioning are attenuated by intensive treadmill exercise. We now routinely use this indicator as a marker to validate the treadmill exercise paradigm. After completion of the exercise brain tissues are analyzed for the expression of striatal DAT and TH proteins comparing MPTP and MPTP + exercise animals. Outcomes from Study 2 on the effect of exercise on DAT protein expression in the striatum are reported in a manuscript (See Fisher et al 2004, Appendix 2). As part of these studies we are analyzing the pattern of expression of TH and DAT mRNA transcripts in nigrostriatal dopaminergic neurons. Studies were carried out using in situ hybridization histochemistry with probes specific for either TH or DAT. The relative degree of mRNA expression is determined by grain counting using computer assisted image analysis of emulsion-dipped sections with cell body staining. The degree of mRNA expression is indicated by the number of grains above neurons. These results indicate that after MPTP-lesioning without exercise there is a reduction in the expression of TH and DAT mRNA when tissues are analyzed at the end of the 28-day exercise period. Analysis of saline + exercise and MPTP + exercise indicates suppression of TH and DAT mRNA expression compared to saline animals without exercise. These results are included in a manuscript now in preparation and soon to be submitted for

review. A preliminary preprint is include as Appendix 4 of this report. The mechanisms responsible for this observation of mRNA suppression are not yet known but studies outlined in Component 1 of this proposal are designed to determine if alterations in glutamatergic neurotransmission contribute to this finding. It is interesting to speculate that the enhanced suppression of striatal DAT protein expression may not take place at the level of its transcription since there is no additional suppression of mRNA seen when comparing MPTP and MPTP + exercise groups as well as the saline and saline + exercise groups. The mechanisms may be involved at the level of DAT and TH translation or axonal translocation. Studies will be carried out in yeas 2 and 3 to analyze alterations in the pattern of expression of CREB and DARPP-32 and their phosphorylated states to determine their contribution to exercise enhanced recovery in the MPTP-lesioned mouse.

Study 3: *The effect of exercise on glutamatergic synapses in the striatum after injury will be determined using ultrastructural immunohistochemical staining with electron microscopy. Pilot data shows altered glutamatergic synapses using immuno-electron microscopy.*

Results from this study using immuno-electron microscopy with an antibody against glutamate are presented in the published manuscript Fisher et al 2004 included as an Appendix in this report. Basically, results of the analysis of striatal tissues derived from saline and MPTP-lesioned mice with or without intensive treadmill exercise indicate normalization of synaptic glutamate levels by exercise. Following MPTP-lesioning there is an elevation in striatal synaptic glutamate compared to non-lesioned mice. However, after treadmill exercise the levels of synaptic glutamate in the MPTP + exercise mice are reduced to near that seen in non-lesioned mice. This indicates that the exercise intervention alters glutamatergic neurotransmission in our model of basal ganglia injury. The most likely source of this glutamatergic neurotransmission is the cortico-striatal pathway. This result provides an important foundation to carryout studies 4 and 5 in this component. Experiments will be pursued to determine if there are alterations in the pattern of expression of AMPA and NMDA receptor subunits and their phosphorylated states in the exercise paradigm. In addition Study 5 will determine if the application of AMPA or NMDA receptor antagonists will alter the degree of glutamate synaptic occupancy we have observed in this Study.

Study 4: *The pattern of expression of subunits for both the NMDA and AMPA receptor subtypes and their phosphorylated state will be determined using western immunoblotting, immunocytochemistry and in situ hybridization histochemistry.*

This Aim is designed to be carried out in Years 2 through 3.5.

Study 5: *We will test the hypothesis that exercise induced neuroplasticity can be attenuated through the administration of either a NMDA or AMPA receptor antagonist. After MPTP-lesioning mice will be subjected to exercise while receiving either the NMDA receptor antagonist MK-801 or the AMPA receptor antagonist GYKI-52466. Behavioral recovery will be compared between groups. Brain tissue will be analyzed for alteration in dopaminergic function (dopamine, DAT and TH expression). Pilot studies show that both glutamate receptor antagonists GYKI-52466 and MK-801 can be administered in this model of MPTP-lesioning.*

This Aim is designed to be pursued in Year 2.5 through 4. However, experiments addressing this Study have been initiated to validate this approach and to prepare for completion of Study 5. Both saline and MPTP-lesioned mice were administered either the AMPA receptor antagonist GYKI-52466 (0.5 or 5.0 mg/kg) and the NMDA receptor antagonist MK-801 (1 mg/kg). Mice were administered

these antagonists starting 4 days after the last injection of MPTP for a period of 30 days. No adverse effects on behavior were observed. The brains from animals in all groups were harvested and analyzed for striatal expression of TH and DAT protein and for expression of TH and DAT mRNA transcripts in nigrostriatal neurons. Data from these experiments show a differential effect of using an AMPA receptor antagonist compared to an NMDA receptor antagonist. For example, comparison of the expression of TH mRNA transcripts in AMPA antagonist treated MPTP-lesioned mice show elevated TH expression in surviving nigrostriatal dopaminergic neurons.

Studies have also been carried out using immuno-electron microscopy with an antibody against glutamate showing no significant alteration in glutamate synaptic occupancy in mice administered the AMPA receptor antagonists GYKI-52466 or the NMDA receptor antagonists MK-801.

Component 2: Pharmacological Enhancement of Neuroplasticity in the MPTP-lesioned Non-Human Primate Model.

***Study 1**: Behavioral assessment of MPTP-lesioned squirrel monkeys administered the dopamine receptor agonist pramipexole.*

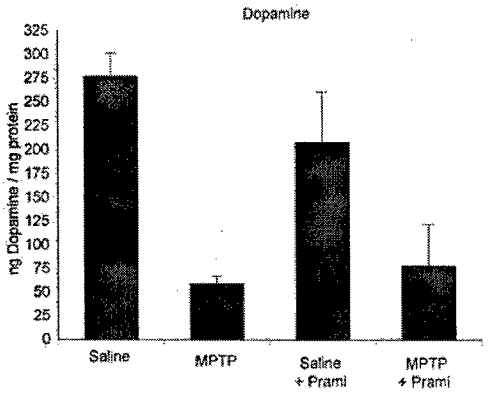
In Year One of this proposal we have carried out the lesioning and behavioral analysis of MPTP-lesioned nonhuman primates in the groups utilizing the 6-week time frame. These are groups 1, 3, 5, and 7 in the Specific Aims Component 2. These squirrel monkeys were lesioned with MPTP in a series of 6 injections of neurotoxicant at a concentration of 2 mg/kg (free-base) administered once every 2 weeks for a total of 12 mg/kg per animal. The typical timeline for this protocol is outlined in Figure 1 showing that acquisition, quarantine (60 days), baseline acclimation and behavioral assessment (30 days), MPTP-lesioning (12 weeks), followed by behavioral assessment in the 6 weeks after the last injection of MPTP. The total time period for this stage is approximately 8 months.

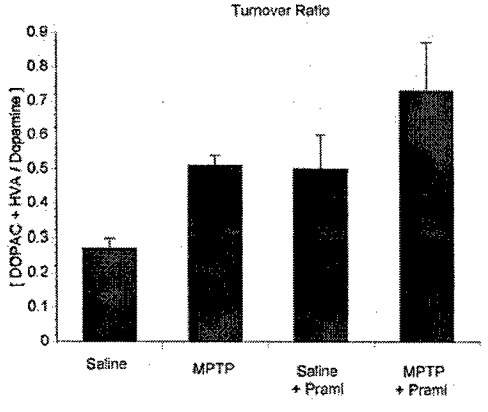
The behavioral assessment of MPTP-lesioned squirrel monkeys with and without pramipexole in the early (6 week) time point has been completed. In study 1 we have added a Sinemet (L-DOPA/ Carbidopa) group for comparison with Pramipexole. This deviation is based on the scientific rationale that L-dopa, unlike Pramipexole, is metabolized and stored by dopaminergic terminals and therefore may have a more direct effect on the regulation of endogenous dopamine production and behavioral recovery and offers an interesting comparison to a compound that is not taken up by terminals. One important outcome in Study 1 was the unexpected induction of dyskinesia in the MPTP-lesioned animals administered Pramipexole. This new finding has not been reported in the literature by other investigators and we are preparing a manuscript reporting this novel finding. This may indicate that Sinemet and Pramipexole may both induced dyskinesias through mechanisms that include the down regulation of the dopamine transporter. Experiments to specifically address this mechanism will be carried out in years 2-3 of this proposal.

The behavioral assessment in this first group of animals carried out up to 6 weeks after the last injection of MPTP did not indicate a significant enhancement of behavioral recovery in either the Sinemet or Pramipexole groups. Long term studies carried out up to 4 months after the last injection of MPTP will determine if there is any enhancement in behavioral recovery that takes place after the 6 week time point, comparing Sinemet + MPTP and Pramipexole + MPTP with the MPTP only group. Animals in this phase of the start are currently undergoing lesioning with MPTP.

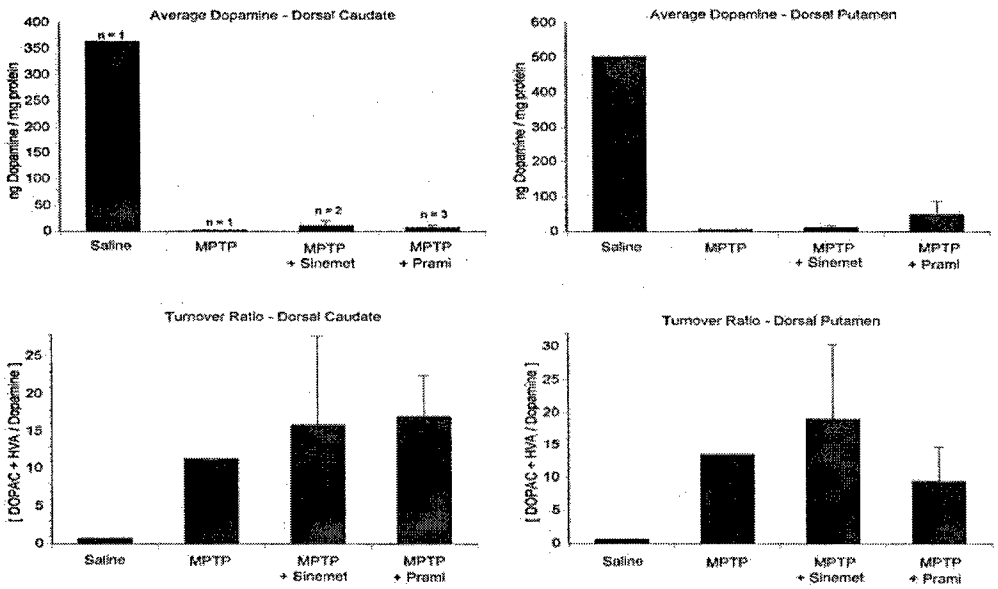
Study 2: Analysis of brain tissue from squirrel monkeys administered pramipexole

As a preliminary study, analysis of dopamine and its metabolites by HPLC was carried out in the MPTP-lesioned mouse. Mice were administered Pramipexole at a concentration of 1 mg/kg 7 days after the administration of MPTP. Animals were dosed once per day for seven days. Brain tissue was harvested and striatal tissue dissected immediately after the last dose of pramipexole. HPLC analysis showed a slight increase in dopamine in the MPTP + Pramipexole group compared to MPTP group. In addition dopamine turnover ratio (dopamine metabolites/ dopamine) was also slightly higher in the MPTP + Pramipexole group compared to MPTP. One mechanism by which dopamine levels are increased with pramipexole may be through the regulation of Tyrosine hydroxylase. This protein and other enzymes important in the biosynthetic pathway of dopamine is being examined in Years 2 and 3.





Analysis of dopamine and its metabolites in the MPTP-lesioned squirrel monkey:
Squirrel monkeys were MPTP-lesioned and then treated one week after the last injection of MPTP with either Sinemet (10 mg/kg twice daily) , or Pramipexole (1 mg/kg twice daily). Animals were treated for three weeks. On each week animals received drug for three days (tue,wed, thurs)) and then saline for four days (fri, sat, sun, mon). Animals were rated each day for parkinsonian features and for dyskinesia. Drug was washed out for 3 weeks and then animals were euthanized. Brain tissue was collected and striatal tissue dissected 6 weeks (1 week monitoring + 3 weeks drug treatment + 2 weeks washout) after MPTP. HPLC analysis showed that pramipexole treated animals had a slight increase in striatal dopamine, especially in the putamen, compared to MPTP or the MPTP + Sinemet groups.



Reportable Outcomes For Year One

Abstracts:

(1) Hogg, E, M. W. Jakowec, K. L. Nixon, A. T. Abernathy, P. Arevalo, B. E. Fisher, M. Liker, and G. M. Petzinger. (2004) Behavioral recovery in the MPTP-lesioned nonhuman primate: Altered dopamine biosynthesis and storage. Society for Neuroscience, San Diego, CA.

Publications:

(1) **Jakowec M.W.**, K. Nixon, E. Hogg, T. McNeill, and G. M. Petzinger. Tyrosine hydroxylase and dopamine transporter expression following 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced neurodegeneration of the mouse nigrostriatal pathway. J. Neurosci. Res. **76** (4) 539-550.

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Presentations:

(1) Petzinger, Giselle MD " Neuroplasticity in the MPTP-lesioned Nonhuman Primate", Plasticity and Repair in Neurodegenerative Disorders, Lake Arrowhead, California, Workshop, Feb19-22, 2004.

(2) Jakowec, Michael PhD " The Role of Exercise in Enhancing Neuroplasticity in the MPTP-lesioned mouse" , Plasticity and Repair in Neurodegenerative Disorders, Lake Arrowhead, California, Workshop, Feb19-22, 2004.

(3) Petzinger, Giselle MD " Enhancing Neuroplasticity in models of Basal Ganglia Injury", Van Der Muelen Symposium, University of Southern California, Keck School of Medicine, April 1, 2005.

Conclusions:

The MPTP-lesioned mouse and squirrel monkey are valuable models for investigating neuroplasticity of the injured basal ganglia. Studies of year one from this proposal indicate that intensive treadmill exercise can enhance motor behavioral recovery and alter the time course of intrinsic neuroplasticity. Our data indicates that alterations in striatal dopamine is not the sole factor responsible for this enhanced recovery. Preliminary data supports the role of the glutamatergic system in exercise related effects on either the injured or normal basal ganglia. Therefore glutamate-dopaminergic interactions may serve as a therapeutic target for enhancing repair.

In the MPTP-lesioned primate we have not observed any enhancement of early (6 week) behavioral recovery through exogenous dopamine replacement therapy either in the form of Sinemet or the dopamine agonist, Pramipexole. Interestingly we have seen a modest increase in striatal (putamen) dopamine in the Pramipexole treated group. Molecular analysis are examining alterations in proteins involved in the biosynthetic pathway of dopamine. One unexpected finding was the development of dyskinesia during treatment with Pramipexole. This behavioral finding has not been previously reported. Dyskinesia was noted to develop at a slightly later time course then that observed in Sinemet treated animals We believe that one possible mechanism for the development of dyskinesia in both Sinemet and Pramipexole treated animals is due to the down regulation of the dopamine transporter which may allow greater diffusion away from the synaptic cleft and a greater interaction with altered glutamate receptors. This hypothesis is currently being investigated using the same studies outlined in our proposal. Studies are underway to examine alterations in long -term behavioral recovery using dopamine replacement therapy.

Appendices: Attached.

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Tyrosine Hydroxylase and Dopamine Transporter Expression Following 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine-Induced Neurodegeneration of the Mouse Nigrostriatal Pathway

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Administration of the neurotoxicant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to C57BL/6 mice targets nigrostriatal dopaminergic neurons, leading to cell death and the depletion of striatal dopamine. After MPTP lesioning in young adult mice, surviving nigrostriatal dopaminergic neurons display robust and reproducible return of striatal dopamine weeks to months after injury. Thus, the mouse provides an excellent model with which to investigate the mechanisms underlying neuroplasticity of the nigrostriatal system following neurotoxic injury. The purpose of this study was to analyze proteins and mRNA transcripts of genes involved in dopamine biosynthesis (tyrosine hydroxylase; TH) and uptake (dopamine transporter; DAT) with regard to time course (7–90 days) after MPTP lesioning. Molecular analysis using immunohistochemistry and Western immunoblotting techniques demonstrated an increase in striatal TH by 30–60 days postlesioning that returned to near-control (prelesioned) levels by 60–90 days. In situ hybridization histochemistry indicated that this increase in TH protein might be due in part to increased TH mRNA expression in surviving nigrostriatal dopaminergic neurons. Analysis of TH protein at 7, 30, 60, and 90 days postlesioning with two-dimensional polyacrylamide gel electrophoresis in conjunction with Western immunoblotting revealed altered TH protein isoforms migrating at isoelectric points different from those of the native isoform. In contrast to TH protein, which returned to prelesioned levels by 60 days, DAT protein analysis showed that increased expression of striatal DAT protein did not return to near-prelesion levels until 90 days postlesioning. These results suggest that TH and DAT may differ in their time course of expression in surviving dopaminergic neurons and may play a role in mediating the return of striatal dopamine.

Key words: MPTP; DAT; substantia nigra; striatum; Parkinson's disease; neurotoxic injury

Recent advances in our understanding of the mammalian brain have revealed that the adult central nervous system (CNS) possesses a tremendous capacity for repair following injury. Repair in the form of neuroplasticity can occur through alterations at a number of different levels within the CNS, including 1) synapse function, 2) synapse number, 3) neuronal phenotype, 4) neuronal and glial specific protein and gene patterns of expression, and 5) neuronal sprouting and branching. In our laboratory, we are interested in understanding the molecular mechanisms involved in neuroplasticity following injury with the neurotoxicant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Administration of MPTP to C57BL/6J mice leads to the destruction of substantia nigra dopaminergic neurons and the depletion of striatal dopamine (Jackson-Lewis et al., 1995). The degree of lesioning is such that approximately 30-35% of the nigrostriatal dopaminergic neurons survive as determined by both section sampling and unbiased stereological counting techniques. These surviving neurons may act as a template mediating recovery either with the nigrostriatal dopaminergic system or other neurotransmitter system affecting basal ganglia function. Immediately after MPTP lesioning, there is a nearly 90% reduction in the level of striatal dopamine. This reduced level of dopamine remains low for the next 30 days but begins to increase and achieves near-prelesion levels 3–4 months after injury (Ricaurte et al., 1986; Bezard et al., 2000). This phenomenon is more pronounced in young adult mice compared with aged mice, in which recovery of striatal dopamine is severely attenuated (Ricaurte et al., 1987b). The reason for this age-

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dependent effect is unclear, but it may share some features similar to the recovery seen in other injury models, such as hypoxia-ischemia and trauma.

The purpose of this study was to investigate the pattern of expression of proteins and mRNA transcripts involved in basal ganglia function, including 1) tyrosine hydroxylase (TH), which is the rate-limiting enzyme in dopamine biosynthesis, and 2) the dopamine transporter (DAT) responsible for dopamine uptake at the synapse. Young adult male C57 BL/6J mice were administered a moderately severe MPTP lesion by using a series of four injections at a concentration of 20 mg/kg, free base, over an 8-hr period. Brain tissue was harvested from the young mice in a time course fashion from 7 through 90 days after MPTP lesioning, and the levels of striatal dopamine, striatal TH and DAT, and TH mRNA transcript were determined. The extent of lesioning was determined by counting the number of substantia nigra pars compacta (SNpc) dopaminergic neurons using unbiased stereological techniques. In addition, because alteration in TH activity through phosphorylation has been proposed as a component of early biochemical compensation, we examined the posttranslational modification of TH protein by using two-dimensional (2-D) polyacrylamide gel electrophoresis in conjunction with Western immunoblotting. An understanding of the molecular mechanisms of intrinsic neuroplasticity within the injured nigrostriatal system may identify new therapeutic targets for the development of means to enhance neuroplasticity or to restore function to surviving dopaminergic neurons.

MATERIALS AND METHODS

Animals and MPTP Lesioning

Young adult (8 weeks old) C57BL/6 mice supplied by Jackson Laboratory (Bar Harbor, ME) were used for all experiments. MPTP (Sigma, St. Louis, MO) was administered in a series of four intraperitoneal injections of 20 mg/kg (free base) at 2-hr intervals for a total of 80 mg/kg. In the 8-week-old mouse, this regimen leads to an approximately 60–70% loss of nigrostriatal neurons (verified by using unbiased stereological methods in our laboratory) and an 80–90% depletion of striatal dopamine levels measured 7 days after lesioning (Jackson-Lewis et al., 1995). Brain tissue was collected for HPLC analysis at postlesioning days 3, 7, 21, 60, 90, and 120. Brain tissue was collected for immunohistochemical, Western immunoblot, and in situ hybridization analyses at postlesion days 7, 14, 30, 60, and 90. In each treatment group and at each time point, there were at least six mice, for a minimum total of 42 mice per time course study. The time course study was carried out in three independent runs. Saline-injected mice served as controls. Preliminary studies have shown that saline control levels did not change through the time course; therefore, collection of control tissues was carried out at injection days 0 and 30, reducing the number of animals needed in these studies.

Determination of Striatal Dopamine Levels and Metabolites by HPLC

Neurotransmitter concentrations were determined according to an adaptation by Irwin et al. (1992) of the method of Kilpatrick and colleagues (1986). The striatum (n = 6 animals at each time point and age) was dissected, and tissues were immediately frozen in liquid nitrogen and stored at −80°C until analysis. Tissues were homogenized in 0.4 N perchloric acid, proteins were separated by centrifugation, and the supernatant was used for HPLC analysis. The protein pellet was resuspended in 0.5 N NaOH and total protein concentration determined using the BCA detection method (Pierce, Rockford, IL). Striatal dopamine was expressed as nanograms dopamine per milligram protein. Statistical analysis was carried out by using a one-way ANOVA with Dunnett's posttest comparing saline (control) treatment with MPTP-lesioned groups.

Immunohistochemical Staining for TH and DAT Proteins

Probes used included antibodies against TH (polyclonal antibody made in rabbit or a monoclonal made in mouse; Chemicon, Temecula, CA), and DAT (monoclonal made in rat; Chemicon). Mice (6 for each treatment group and time point) designated for immunohistochemistry were administered pentobarbital (100 μl of 40 mg/ml, i.p.), then perfused transcardially with 50 ml of ice-cold 0.9% saline, followed by 50 ml of 4% paraformaldehyde/phosphate-buffered saline, pH 7.2 (called *PFA/PBS*). After perfusion, brains were quickly removed, immersion fixed in 4% PFA/PBS at 4°C for 24–48 hr, then cryoprotected in 20% sucrose for 24–48 hr. Brains were then quickly frozen in isopentane on dry ice; cut into 30-μm-thick sections, placed in phosphate-buffered saline, pH 7.2; and used immediately for immunohistochemical staining. Sections were rinsed in TBS (50 mM Tris, pH 7.2, with 0.9% NaCl); quenched in 10% methanol/3% H_2O_2/50 mM Tris, pH 7.2; blocked in 4% normal serum; exposed to antibody (concentration 1:1,000) for 48 hr at 4°C; rinsed in TBS; and then exposed to secondary antibody made against the species of the primary antibody (using the ABC Elite Kit; Vector, Burlingame, CA). Antibody staining was visualized by development in 0.1% diaminobenzoic acid/3%H_2O_2. To ensure that differences in staining intensity were in fact due to differences in antigen expression, multiple sections from each treatment group and time point were concurrently used under identical staining conditions. Specificity of antibody probes was verified by methods that eliminated staining, including 1) omitting primary antibody, 2) omitting secondary antibody, and 3) omitting both primary and secondary antibodies.

Western Immunoblotting

Western immunoblotting was used to determine the relative striatal expression of TH and DAT protein. The immunoblotting technique was previously described (Jakowec et al., 1995a, 1998, 2001). In total, six mice were used at each time point. After decapitation, tissue was dissected from the striatum or ventral mesencephalon and homogenized in buffer [25 mM Tris, pH 7.4, 1 mM EDTA, 100 μM phenylmethylsulfonyl fluoride (PMSF)]. Protein concentration was determined by the BCA method (Pierce). Equal amounts of protein (25 μg) were separated by the method of Laemmli (1970). Proteins were transferred to nitrocellulose filters by electroblotting in Towbin buffer (Towbin et al., 1979). Filters were blocked in TS-Blotto (50 mM Tris, pH 7.4, 0.9% NaCl, 5% nonfat milk), then

primary antibody (1:2,000), and exposed to secondary antibody and visualized by chemiluminescence (Pierce). Filters were apposed to film (Hyperfilm ECL; Amersham, Arlington Heights, IL) and processed in X-Omat developer. Images were scanned into a computer using a Personal Laser Densitometer (Molecular Dynamics, Sunnyvale, CA), and the intensity of bands was determined by using computer-assisted image analysis (NIH Image). The intensities of bands from Western blot autoradiographs were expressed as relative optical density. The relative optical density of each treatment group was expressed as a percentage of the day-7 saline-injected group. Group comparisons were made by using one-way ANOVA with Bonferoni correction to determine statistical significance or effect size (meaningful difference; Thomas et al., 1991).

Unbiased Stereological Counting of SNpc Dopaminergic Neurons

The number of nigrostriatal dopaminergic neurons in the SNpc was determined by using unbiased stereology with the computer-imaging program BioQuant Nova Prime (Bioquant Imaging, Nashville, TN) and an Olympus BX-50 microscope equipped with a motorized stage and CCD camera. Brain tissue was prepared from three mice in each group as described for immunohistochemical staining. Tissue was sliced at 30 μm thickness and every third section collected and stained for TH immunoreactivity and counterstained for Nissl substance. The SNpc was delineated from the rest of the brain based on TH immunoreactivity. Each ventral mesencephalon section was viewed at low magnification (×10 objective) and the SNpc outlined and delineated from the ventral tegmental-immunoreactive neurons by using the third nerve and cerebral peduncle as landmarks. Neurons were viewed at high magnification (×80 objective) and counted if they displayed TH immunoreactivity and had a clearly defined nucleus, cytoplasm, and nucleolus. The total number of SNpc dopaminergic neurons was determined based on the method of Gunderson (1987).

Two-Dimensional Polyacrylamide Gel Electrophoresis/ Western Immunoblotting

Striatal tissue was homogenized in buffer (25 mM Tris-HCl, pH 7.4, 1 mM EDTA, 100 μM PMSF, and 0.5% Triton X-100), and the total protein concentration was determined by the BCA method (Pierce). A total of 50 μg of protein was resuspended in isoelectric focusing buffer (8 M urea, 2% NP-40, 0.8% ampholytes, pH 3–10, 2% β-mercaptoethanol, and 100 μM PMSF) and placed over a pH 3–10 NL IPG strip (Pharmacia, Piscataway, NJ). Proteins were separated based on their isoelectric point by using the IPGphor (Pharmacia) for 60,000 volt-hours. After isoelectric focusing, the IPG strip was placed over a 12.5% polyacrylamide gel, and proteins were separated based on their molecular weight (Laemmli, 1970). A "standards well" to one side of the 2-D strip well was spiked with 25 μg of total protein (from the original tissue homogenate) to identify the TH protein migration point. After protein separation, the gel was subjected to identical conditions as described for the 1-D polyacrylamide gel electrophoresis/ Western immunoblotting (see above). Sample gels (after the second-dimension separation) were subjected to silver staining to visualize total protein pattern, ensuring adequate separation and quality (Harrington and Merril, 1984).

In Situ Hybridization Histochemistry

In situ hybridization histochemistry (ISHH) was used to determine the relative expression of TH mRNA in the midbrain. Methods for this technique have previously been described (Jakowec et al., 1995b, 1998). At least six mice in each treatment group and time point were used. After decapitation, brain tissue was quickly removed and frozen in isopentane on dry ice. Sections were cut at 14 μm thickness on a Leica Jung 1850 cryostat, thaw mounted onto poly-L-lysine-coated microscope slides, dried on a 55°C slide warmer, and fixed in 4% paraformaldehyde/phosphate-buffered saline. Sections were dehydrated in successive ethanol washes (30%, 60%, 80%, 95%, and 100%), deprotonated in triethanolamine/acetic anhydride, delipidated in chloroform, and dehydrated in ethanol. A ^{35}S-ribonucleotide probe was generated from a cDNA clone of the TH gene (Grima et al., 1985; Chesselet et al., 1987) by using an in vitro transcription kit from Promega (Milwaukee, WI). Tissue sections were exposed to hybridization buffer containing 4× standard sodium citrate (SSC; 1× SSC is 0.15 M NaCl, 0.015M sodium citrate, pH 7.0), 50% formamide, 1× Denhardt's solution, 250 μg/ml tRNA, 500 μg/ml salmon sperm DNA, 10% dextran sulfate, with 1×10^6 cpm of radiolabeled ribonucleotide probe. After 24 hr, tissue sections were washed in 2× SSC/50% formamide/0.1% β-mercaptoethanol at 37°C for 30 min, then in 20 μg/ml RNase in 0.5 M NaCl/10 mM Tris, pH 7.4, at 37°C for 45 min, then in 2× SSC/50% formamide/0.1% β-mercaptoethanol at 60°C for 1 hr, then in 0.1× SSC/0.1% β-mercaptoethanol at 65°C and rinsed in ethanol. Slides were placed against high-resolution film (Hyperfilm β-Max; Amersham) with radioactive standards (Amersham) for 3–7 days. The relative optical density above the SNpc from 20 different sections from groups of animals injected with saline or MPTP (at postlesioned days 7, 30, 60, and 90) was determined with a Molecular Dynamics Personal Densitometer. To minimize potential sources of variation between different experimental runs, slides from the different treatment groups and time points were processed concurrently using identical hybridization cocktail, probe concentration, probe preparation, wash regimen, and length of film exposure.

RESULTS

Striatal Dopamine Levels

The time course of striatal dopamine return was determined by HPLC analysis (Fig. 1). The regimen of MPTP administration used in these studies resulted in a depletion of striatal dopamine level at 3 days postlesioning to 14% ± 6% (23.4 ± 7.8 ng dopamine/mg protein; mean ± SEM) of prelesioned levels (where prelesioned levels of 100% were equal to 155.9 ± 17 ng of dopamine/mg protein). A slight decline (but not statistically significant compared with the level at 3 days post-MPTP) was seen at 7 days post-MPTP (10.3 ± 5 ng dopamine/mg protein). At 21 days postlesioning, dopamine levels remained low (22% ± 9% of control equal to 34.3 ± 12.5 ng dopamine/mg protein) but showed an

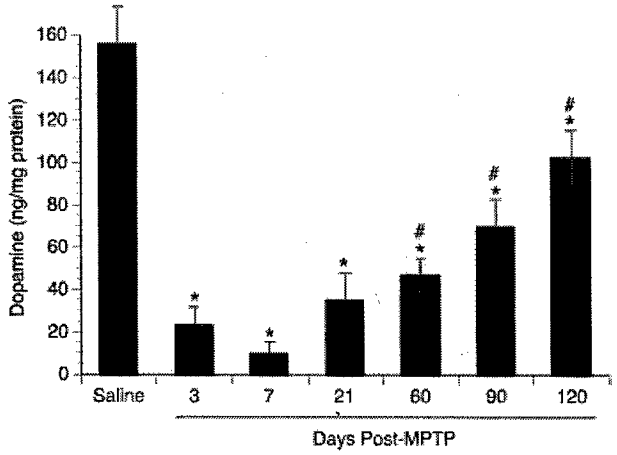

Fig. 1. Time course of striatal dopamine return after MPTP lesioning in young and old mice. The concentration of dopamine in tissue dissected from the midstriatum was determined by HPLC analysis at post-MPTP lesioning days 3, 21, 60, 90, and 120 days. Mice administered saline acted as control (day 0 time point). The concentration of dopamine is expressed as the percentage of control (saline injected) level. Asterisks represent statistical significance compared with the saline group and the number signs indicate statistical significance compared with the 7 day post-MPTP lesion groups ($P < .05$). Error bars are SEM.

increase by postlesioned day 60 (30% ± 6% of control equal to 46.8 ± 5.0 ng dopamine/mg protein). By postlesion day 90 the level of dopamine was at 45% ± 10% of control (equal to 70.2 ± 8.0 ng dopamine/mg protein) and reached 67% ± 9% of control (102.9 ± 8.0 ng dopamine/mg protein) by 120 days postlesioning. All post-MPTP lesioning dopamine levels were statistically significantly different ($P < .05$) from prelesioned (saline) control levels (at day 0). The levels of dopamine at post-MPTP lesioning days 60, 90, and 120 were statistically significantly different compared with the 7-day time point ($P < .001$). The level of dopamine in saline-treated animals did not change significantly during the time course of these studies.

Degree of MPTP Lesioning

The degree of nigrostriatal cell loss in the SNpc following MPTP administration was determined by using an unbiased stereology counting technique. Nigrostriatal dopaminergic neurons were stained for TH immunoreactivity (TH-ir) and counterstained for Nissl substance. The saline-injected mice had a total of 23,670 (SEM = 1,250; n = 2) TH-ir neurons in the SNpc, whereas mice analyzed 7 and 30 days after MPTP lesioning had a total of 7,410 (SEM = 304; n = 2) TH-ir neurons. This represented a total decline of 69% in the number of SNpc dopaminergic neurons. Representative sections through the midbrain are shown in Figure 2, indicating that there is no significant change in the number of TH-ir neurons during the first month post-MPTP lesioning. The degree of SNpc dopaminergic neuron cell loss determined by using a lesioning regimen of four injections of 20 mg/kg MPTP (free base) is similar to that previously reported with a sampling technique to count TH-ir neurons (Jackson-Lewis et al., 1995). These results indicate that there is no substantial increase in TH-ir neurons during recovery from MPTP lesioning.

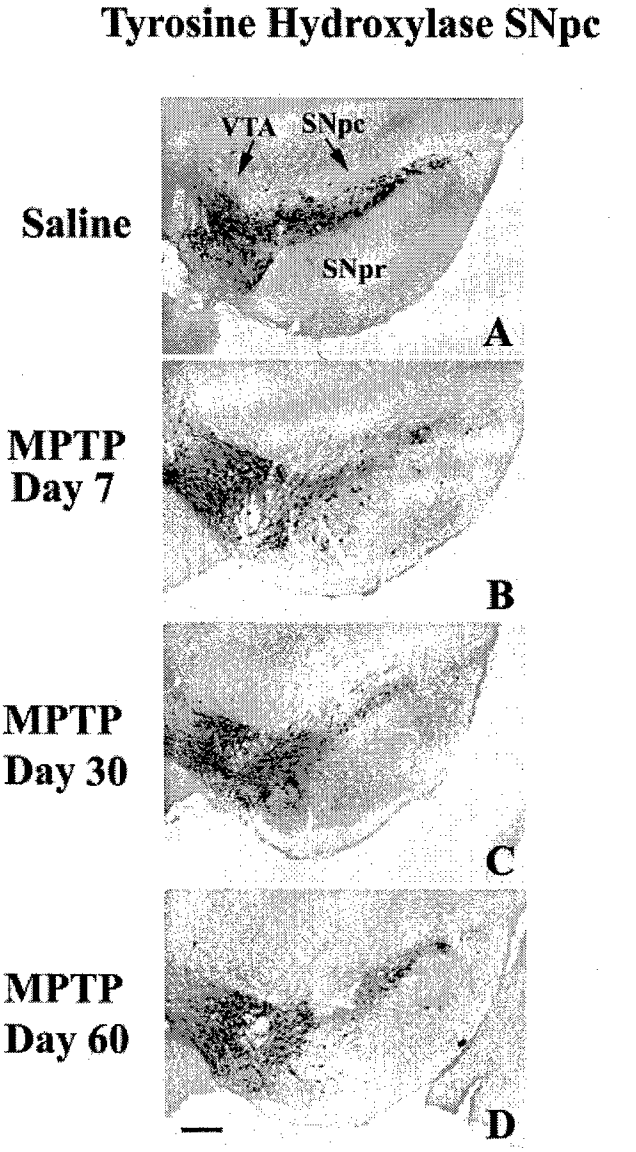

Fig. 2. Degree of MPTP lesioning in the substantia nigra pars compacta. Coronal sections stained for TH-ir at the level of Bregma –3.20 showing representative images from the midbrain of mice saline injected (**A**), 7 days post-MPTP lesioning (**B**), 30 days post-MPTP lesioning (**C**), and 60 days post-MPTP lesioning (**D**). Unbiased stereological methods were used to show that the MPTP lesioning regimen used in these studies led to an approximately 70% loss of nigrostriatal dopaminergic neurons. Scale bar = 100 μm.

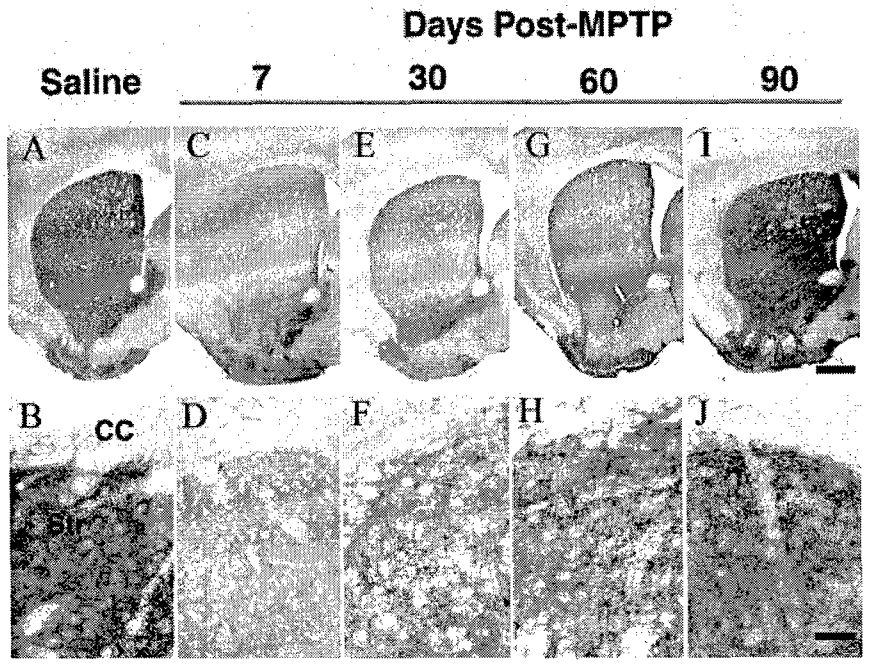

Fig. 3. TH protein expression in the striatum after MPTP lesioning. Immunohistochemistry analysis using an antibody against TH protein demonstrated increased expression that correlated with the return of striatal dopamine. Coronal sections at the level of the midstriatum (Bregma 1.00) were stained for TH protein and images made at low magnification (20×; upper panels) or high magnification (400×; lower panels). In animals administered saline, intense TH-ir was seen throughout the striatum (dark staining; **A**), and a high-magnification image (**B**) taken from the most dorsal aspect of the striatum adjacent to the corpus callosum (cc) showed a thick network of TH-positive fibers. At 7 days post-MPTP lesioning (**C**), there was a severe depletion of TH-ir, and at high magnification (**D**) only small numbers of positive immunoreactive fibers were evident. At 30 days post-MPTP lesioning (**E**), there was an increase in TH-ir in the striatum, with large fibers evident (**F**). At 60 days post-MPTP lesioning, there was a further increase in TH-ir (**G**), with increased fiber density (**H**). At 90 days, TH-ir increased (**I**) relative to 60 days, and the TH-ir fiber density appears to consist of both thick and thin fibers (**J**) forming a network similar to that seen in saline-injected animals but with an overall lower level of immunoreactivity. Scale bar in I = 100 μm for A,C,E,G,I; bar in J = 10 μm for B,D,F,H,J.

Analysis of TH Expression

The pattern of expression of TH protein in the striatum was determined by using both immunohistochemical staining (to examine anatomical distribution of TH-ir) and Western immunoblotting (to compare relative TH protein levels at the different time points). Immunohistochemical staining of coronal sections at the level of Bregma 1.00 showed intense fibrous TH immunoreactivity throughout the striatum and olfactory tubercle of saline-injected mice (Fig. 3A,B). At 7 days post-MPTP lesioning, the degree of TH-ir was greatly reduced, with only a small number of immunopositive fibers within the striatum (Fig. 3C,D). At 30 days post-MPTP lesioning, the TH-ir increased, with more intense thick staining fibers observed (Fig. 3E,F). The degree of TH-ir protein increased through day 60, with greater staining observed in both large- and small-diameter fibers (Fig. 3G,H). At post-MPTP lesioning day 90, TH-ir was greater than that observed at day 60 but was still slightly below prelesioning staining levels (Fig. 3I,J).

Western immunoblot staining was performed on isolated striatal tissues to compare the relative amount of TH protein at time points after MPTP lesioning (Fig. 4). The day-7 saline-injected group was designated as 100%, and for comparison all other groups were normalized against this, with values expressed as mean ± SEM. Analysis of the relative amount of TH protein in the saline-injected group showed no significant change between day 7 and day 30 (100% vs. 102% ± 10%, respectively). However, 7 days following MPTP lesioning, the relative amount of TH-immunopositive protein declined to 28% ± 5% of the level in saline-injected mice ($P < .001$). At 30 days post-MPTP lesioning, TH-ir increased to 45% ± 12% ($P < .004$) of saline control levels. For TH protein levels at postlesioning days 60 and 90, the relative amount rose to 60% ± 14% and 74% ± 12% of that of saline-injected mice, respectively, and was not significantly different from that of the saline group.

Analysis of TH mRNA in the ventral mesencephalon was carried out by using ISHH. Images made at the level of the SNpc (Bregma −3.40) following ISHH with a ^{35}S-radiolabeled probe against TH mRNA showed intense grain density above both the SNpc and VTA (Fig. 5) in saline-injected animals. For comparison, the relative

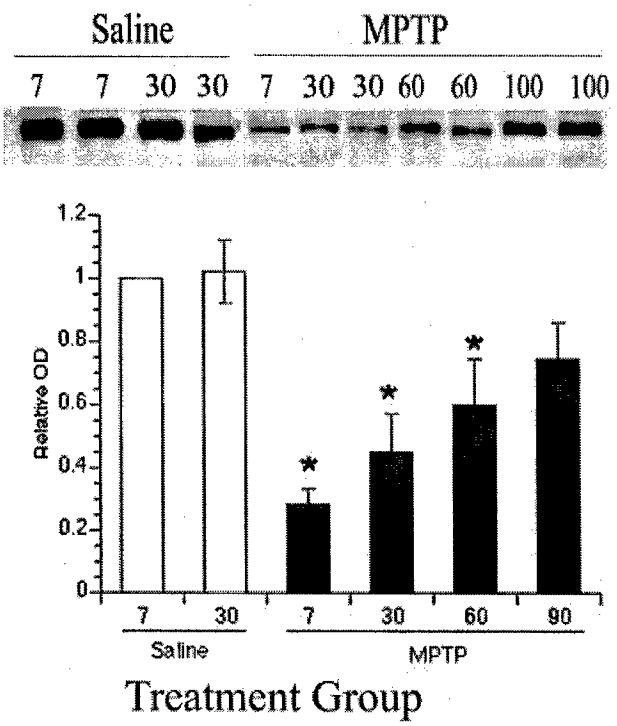

Fig. 4. Western immunoblot analysis of striatal TH protein. Analysis was carried out on tissue derived from the midstriatum of mice injected with either saline or MPTP. The upper panel shows a representative Western blot from a single experiment with equal loading of total protein. Data are expressed relative to saline-treated animals as optical density of autoradiographic bands. The relative amount of TH protein in saline-injected animals was arbitrarily set as 100%, and results from other groups were normalized against it. No difference in the relative amount of TH protein was seen after saline injection at either 7 or 30 days. MPTP lesioning resulted in a depletion of striatal TH protein at 7 days to 28% ± 5% of saline control level, increasing slightly at day 30 to 45% ± 12% of control level. A significant increase in TH protein was seen between day 30 and day 90 to 60% ± 14% and 74% ± 12% of prelesion control, respectively. Asterisks represent statistical significance compared with the saline group ($P < .05$). Error bars are SEM.

optical density for the saline group was set to 100% and the relative optical density for all other groups normalized against it. At 7 days postlesioning, the intensity of grains above the SNpc was reduced by 76% compared with saline control (from 100% ± 12.4% in the saline group to 24.0% ± 4.0% at post-MPTP lesioning day 7; Fig. 5B,C). At 30 days postlesioning, there was a slight increase in TH mRNA labeling to 31.6% ± 3.9% of control that further increased at 60 days postlesioning to 40.4% ± 5.0% of saline control (Fig. 5D,E) and by 90 days postlesioning (Fig. 5F) labeling returned to a level not significantly different from prelesioning levels (100.4% ± 8.4%). The level of mRNA increased to near-prelesion levels by day 90 post-MPTP lesioning, even though the total number of SNpc dopaminergic neurons remained near 30% of prelesion levels.

Two-dimensional PAGE separates proteins in the first dimension based on their isoelectric point and in the second dimension based on their molecular weight. Figure 6 shows the results of analysis of striatal TH protein from saline and post-MPTP lesioning days 7, 30, 60, and 90. In saline-treated mice, TH-ir is localized to the native isoelectric point (pI = 5.8). At 7 days postlesioning, the native isoform of TH-ir is nearly absent, with a small amount of TH-ir protein migrating to a new isoelectric point (Fig. 6B). At 30 and 60 days postlesioning, the amount of TH-ir protein at the new isoelectric point continues to increase as well as the TH-ir protein at the native isoelectric point (Fig. 6C,D). By 90 days postlesioning, the remaining TH-ir protein shifted toward the native isoelectric point (Fig. 6E). The apparent molecular weight (56 kDa) of the TH-ir band does not appear to change significantly during the time course of recovery.

Analysis of DAT

The pattern of expression of the DAT protein was determined by using both immunohistochemistry (to examine anatomical distribution) and Western immunoblotting (to compare relative TH protein levels at the different time points) at the level of the midstriatum (Bregma 1.00). Immunohistochemical staining showed intense DAT-ir throughout the midstriatum (Fig. 7A,B). At 7 days postlesioning, the level of DAT-ir was greatly reduced compared with that of saline-injected mice (Fig. 7C,D). At postlesion days 14 (Fig. 7E,F) and 30 (Fig. 7G,H), there was an increase in DAT-ir compared with postlesion day 7 because of an increase in the DAT-ir fiber density. At postlesion days 60 (Fig. 7 I,J) and 90 (Fig. 7K,L), the level of DAT-ir continued to increase relative to the 30-day MPTP lesion time point and resembled the level of DAT-ir seen in saline-injected mice.

Western immunoblotting was used to determine the relative expression of DAT protein in striatal tissue during the time course of recovery. For a relative comparison, the level of DAT protein in striatal tissue homogenates from saline-injected mice was set as 100%, and the value for all other groups was normalized to it (see Fig. 8). There was no change in the relative amount of DAT protein in saline-injected mice at days 7 and 30 (100% vs. 90% ± 5%, respectively). A significant ($P < .0001$) depletion of DAT protein to 34% ± 5% of that of saline-injected mice was seen at 7 days postlesioning. At 30 days postlesioning, DAT protein increased to 44% ± 6% of that of the saline-injected group ($P < .0001$). DAT-ir continued to increase through postlesion day 60 (to 62% ± 7% of that in the saline group; $P < .001$) and day 90 (78% ± 13% vs. saline; not significantly different). Western immunoblotting with tissues from the ventral mesencephalon also showed a reduction in DAT-ir at days 7 and 30 postlesioning that returned to nearly the saline-injected levels from 60 days onward (data not shown).

DISCUSSION

After neurotoxic injury with MPTP in the C57 BL/6 mouse, surviving nigrostriatal dopaminergic neurons

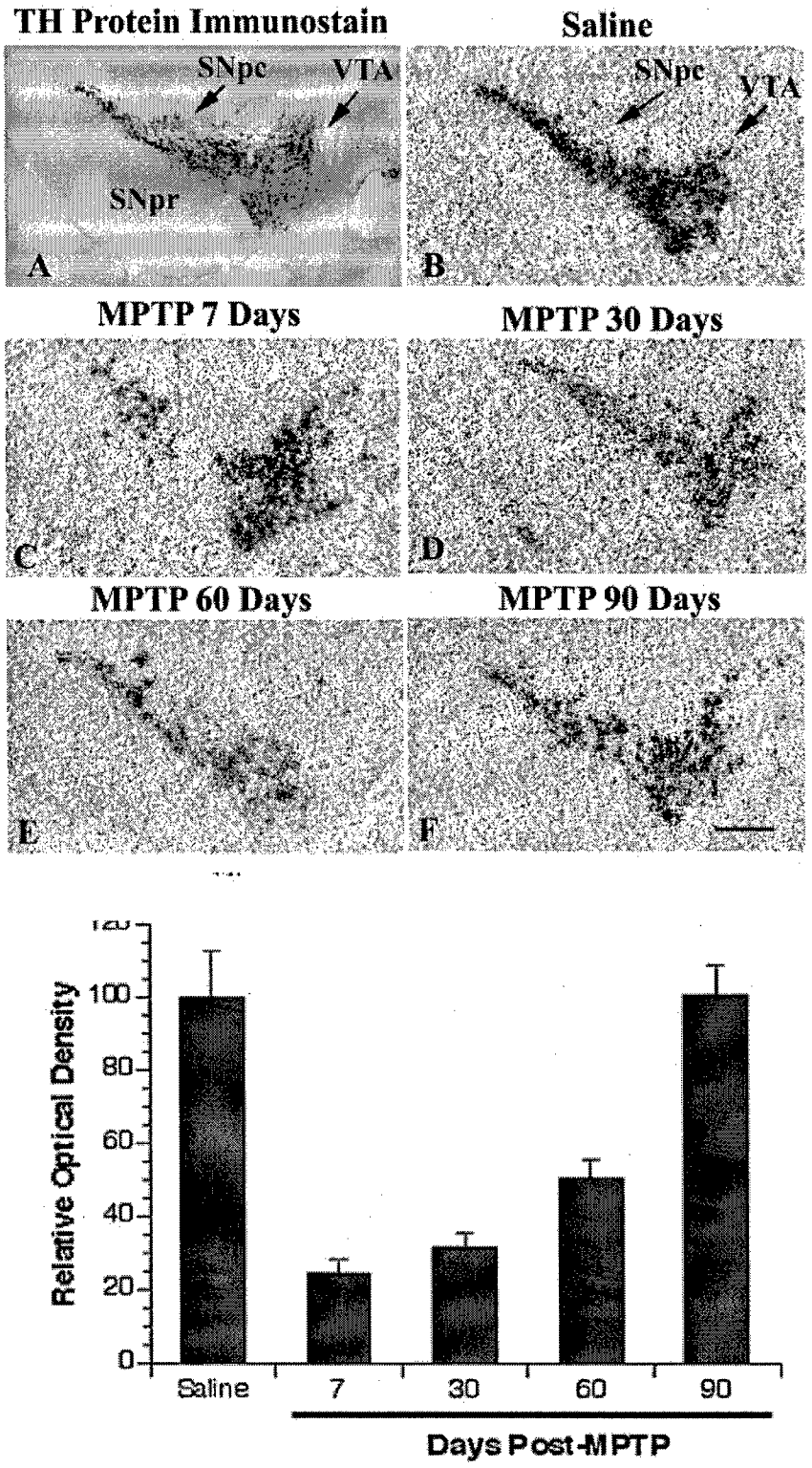

Fig. 5. TH mRNA expression after MPTP lesioning. In situ hybridization histochemistry was carried out on coronal tissue sections at the level of the midsubstantia nigra (Bregma −3.20) from saline-treated and post-MPTP lesioning days 7, 30, 60, and 90. **A:** Immunohistochemical staining against TH protein identified the substantia nigra pars compacta (SNpc) and ventral tegmental area (VTA), where TH-ir cell bodies and their terminals stain dark. **B:** In saline-injected animals, the expression of TH mRNA was robust, with a high density of autoradiographic grains seen above the SNpc and VTA. **C:** At 7 days after MPTP lesioning, there was a loss of grain intensity above the SNpc and only a slight reduction in intensity above the VTA. **D:** The SNpc grain intensity remained low through day 30. **E:** At 60 days after MPTP lesioning, there was increased grain density in the SNpc. **F:** At 90 days after MPTP lesioning, there was an increase in the autoradiographic grain intensity in the SNpc and VTA. MPTP lesion days 7, 30, and 60 are statistically significant (at $P < .001$) compared with saline controls. The optical density was not significantly different between saline and post-MPTP lesion day 90. Scale bar = 100 μm.

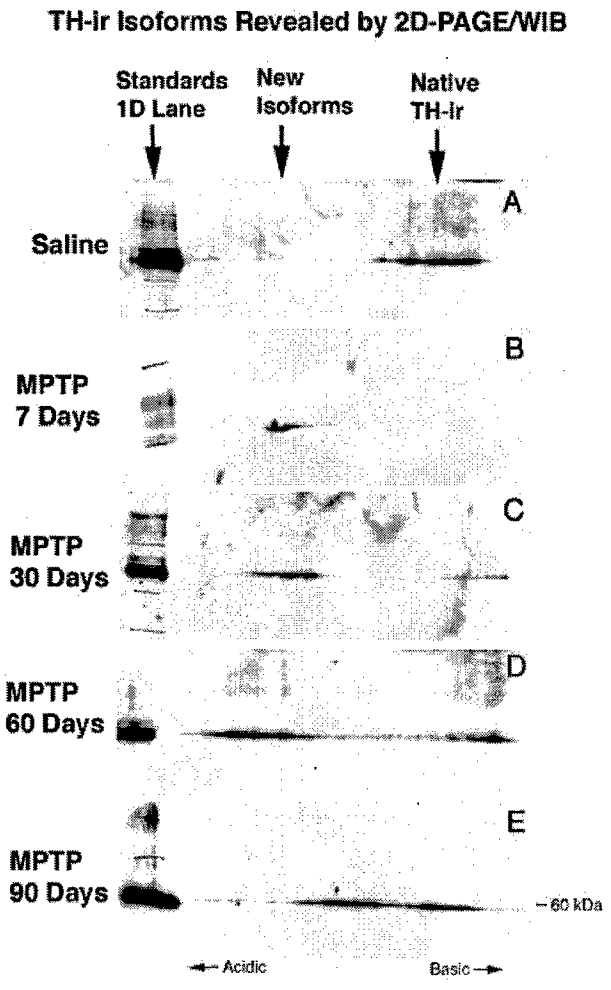

Fig. 6. TH protein expression analyzed by 2-D PAGE. Protein extracts from the striatum of both saline and MPTP-lesioned animals were examined using 2-D PAGE in conjunction with Western immunoblotting. The protein standards lane was "spiked" with striatal protein extract to locate TH-ir. **A:** Saline-injected animals showed a high degree of TH-ir as a single major band in the 1-D lane that was present in a localized cluster of several equivalent-molecular-weight spots according to 2-D separation. **B:** Seven days after MPTP lesioning, the level of TH-ir was significantly reduced as detected by both 1-D and 2-D separation. Small spots of reactivity were evident toward the acid end of the 2-D PAGE. **C:** At 30 days after MPTP lesioning, there was a return of TH-ir as seen on 1-D PAGE. By 2-D PAGE, the increase in intensity was greater at the new isoform position, with only a slight increase in intensity at the native 2-D PAGE position. **D:** At 60 days postlesioning, TH-ir continued to increase at the position of both the new isoform and the native TH position. **E:** At 90 days postlesioning, immunolabeling corresponded to both the native and the new isoform positions.

display robust and reproducible neuroplasticity. Young mice (8 weeks of age) showed return of striatal dopamine as determined by HPLC to near-prelesion levels 3–4 months after injury. Our results confirm the return of striatal dopamine described in earlier studies (Ricaurte et al., 1987b). The degree of nigrostriatal injury may only delay intrinsic neuroplasticity, in that the return of striatal TH protein or TH fiber density has been demonstrated in both mild and severe MPTP lesioning regimens (Bezard et al., 2000). The age-dependent effects of MPTP have also been demonstrated in mice and in nonhuman primates (Ricaurte et al., 1987a; Song and Haber, 2000) as well as with 6-hydroxydopamine in rodents (Onn et al., 1986; Blanchard et al., 1995, 1996).

One mechanism responsible for mediating the return of striatal dopamine in surviving nigrostriatal dopaminergic neurons is through increased expression of TH protein, the rate-limiting enzyme in dopamine biosynthesis (Hefti et al., 1980; Onn et al., 1986; Blanchard et al., 1995, 1996). Based on both immunohistochemical staining for TH protein in the striatum and Western immunoblot analysis of TH protein after MPTP lesioning, the increase in striatal dopamine correlates with the increase in striatal TH protein. Other reports have also examined the return of striatal TH protein following neurotoxic injury of the nigrostriatal pathway. In general, TH protein return occurs late (months) after 6-OHDA lesioning (Onn et al., 1986; Blanchard et al., 1995, 1996) or after MPTP lesioning in both the mouse (Bezard et al., 2000) and the nonhuman primate (Song and Haber, 2000). The time course for the return of striatal TH protein is dependent on the degree of lesioning (a threshold effect); severe lesioning fails to demonstrate return until several months postlesioning, whereas mild lesioning shows recovery within weeks (Bezard et al., 2000; Finkelstein et al., 2000; Song and Haber, 2000). Changes in TH mRNA expression after nigrostriatal injury have been less clear. For example, there are reports of mRNA increasing (Blanchard et al., 1995, 1996) and reports of it decreasing or remaining unchanged (Pasinetti et al., 1989, 1992; Sirinathsinghji et al., 1992). These discrepancies may be due to differences in degree of lesioning, time course, mode of analysis, and rodent strain used. In human parkinsonism, there is a decrease in TH protein and mRNA relative to control brains, which may reflect the severity of disease progression (Javoy-Agid et al., 1990; Kastner et al., 1993). Results from our studies indicate that the remaining nigrostriatal neurons in the midbrain increase mRNA levels such that the absolute level of mRNA labeling as determined by in situ hybridization histochemistry returns to near-prelesioned levels by 90 days. It appears that the remaining cells (30% of saline control) significantly increase the per cell level (possibly by a threefold increase) of the TH mRNA transcript. Studies are underway to quantify this observation more accurately.

In this report, we describe an early and persistent posttranslational modification of TH protein correlating with the return of striatal dopamine after MPTP lesioning in the C57 BL/6 mouse. Our results using 2-D PAGE indicated expression of an altered isoform of TH protein, having a shift in its isoelectric point with little detectable change in the apparent gross molecular weight (Gutierrez

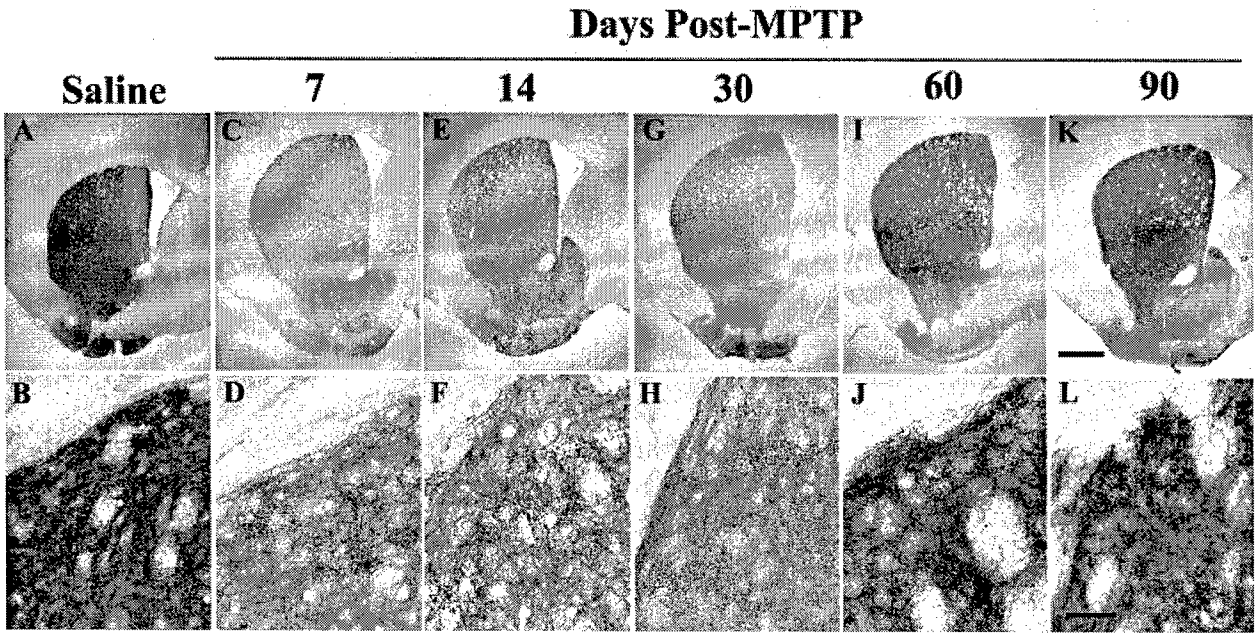

Fig. 7. Immunocytochemical analysis of DAT protein in the striatum after MPTP lesioning. Tissue sections in the coronal plane through the midstriatum (Bregma 1.00) were stained with an antibody against the DAT protein. Images were made at low magnification (20×; upper panels) to show an entire coronal hemisphere and at high magnification (400×; upper panel) from the uppermost dorsal-lateral quadrant of the striatum apposing the corpus callosum). Saline-injected mice showed intense DAT-ir throughout the striatum (**A**) and as a thick fibrous web (**B**). MPTP administration results in the depletion of DAT-ir as seen at postlesion day 7 (**C,D**). DAT-ir remained low at day 14 (**E,F**), with thick processes stained. DAT-ir increased slightly at day 30 (**G,H**) and continued to increase at day 60 (**I,J**) and day 90 (**K,L**). Scale bar in K = 100 μm for A,C,E,G,I,K; bar in L = 10 μm for B,D,F,H,J,L.

et al., 1988). This altered isoform, which was the predominant form of TH protein, occurred early (at 7 days postlesioning) and persisted (for at least 90 days postlesion). Although the molecular basis for this altered form of TH protein has yet to be determined, reports in the literature suggest phosphorylation by protein kinases, including protein kinase A, CAM-protein kinase II, and ERK (MAP kinase) as likely candidates (Campbell et al., 1986; Le Bourdelles et al., 1991; Sutherland et al., 1993; Alterio et al., 1998). Posttranslational phosphorylation of TH protein (at serine residues 8, 19, 31, and 40) leads to an increase in TH enzymatic activity, protein stability, and translocation (Zigmond et al., 1989). This increases the affinity of TH for its cofactor (biopterin) and decreases its feedback inhibition by dopamine (Zigmond et al., 1989; Haycock and Haycock, 1991; Haycock et al., 1998; Fitzpatrick, 1999). We cannot exclude nitrosylation of tyrosine residues in TH as an early effect of MPTP lesioning (Ara et al., 1998). Future studies will examine the effect of this posttranslational modification on the activity of TH enzyme to determine whether alteration in Vmax (Zigmond et al., 1989; Fitzpatrick, 1999) or "homospecific activity" (defined as TH enzymatic Vmax relative to TH protein content; Lloyd et al., 1975; Acheson and Zigmond, 1981; Zigmond et al., 1984; Onn et al., 1986; Mogi et al., 1988) are altered and whether these alterations persist throughout the recovery process.

Another mechanism for increasing synaptic availability of dopamine is through reduced uptake (Rothblat and Schneider, 1999). The dopamine transporter (DAT) protein is responsible for the reuptake of dopamine from the extracellular space for vesicular repackaging in the presynaptic terminal (Hoffman et al., 1998). Our results indicated an early decline of DAT protein (7 days after MPTP lesioning), with a gradual return to 80% of the prelesioned level by 90 days. A consequence of the early decline of DAT protein is to increase the effective synaptic concentration of dopamine (Eberling et al., 1999; Kilbourn et al., 2000). However, it is more likely that the initial decline in DAT protein is due simply to the loss of nigrostriatal fibers as a consequence of MPTP lesioning. The later increase in DAT protein correlated with the return of striatal dopamine and striatal TH protein and may serve as a marker of terminal recovery (sprouting and branching) after 30 days postlesioning. These results are consistent with the expression of genes and proteins involved in neuronal sprouting and support a morphological component to recovery. Studies in our laboratory and the laboratories of others have shown that expression of the growth-associated protein of 43 kDa (GAP-43) involved in neuronal sprouting increases after 30 days post-MPTP lesioning (Jakowec, 1999; Song and Haber, 2000).

Other factors that may influence the return of striatal TH protein include 1) neurogenesis within the substantia

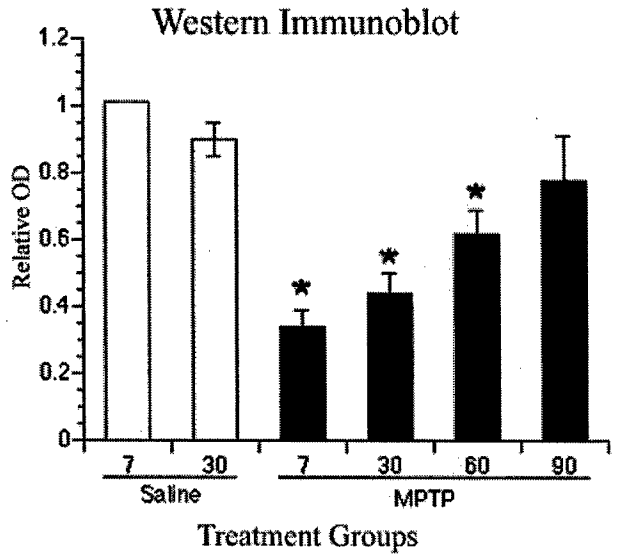

Fig. 8. Western immunoblot analysis of DAT protein in the striatum after MPTP lesioning. The expression of DAT protein was determined by using Western immunoblotting on tissue from the striatum of either saline- or MPTP-injected animals. For relative comparison, the day-7 saline-injected group was arbitrarily set at 100%, and the results from all other groups were normalized against it. There was no significant difference between saline groups at days 7 and 30. After MPTP lesioning, there was a reduction in the level of DAT protein to 34% ± 5% of control. DAT protein levels increased at 30 days postlesioning to 44% ± 6% of saline control and continued to increase at 60 and 90 days to 62% ± 7% and 78% ± 13% of control, respectively. Asterisks represent significant different from saline group ($P < .05$). Error bars show SEM.

nigra as demonstrated by bromodeoxyuridine (BrdU) labeling (Kay and Blum, 2000), 2) conversion of neurons intrinsic to the basal ganglia to a dopamine-producing phenotype (Betarbet et al., 1997), or 3) dopaminergic innervation from areas other than the substantia nigra (Ho and Blum, 1998). In this study, there was no detectable change in the number of SNpc neurons with recovery as measured with unbiased stereological techniques to determine the number of remaining dopaminergic neurons. This finding is supported by studies from other investigators (Bezard et al., 2000). Therefore, it appears that nigrostriatal dopaminergic neurons that survive the MPTP lesioning increase TH and DAT protein and may repopulate the striatum with axonal regrowth and branching.

Understanding the inherent neuroplasticity of the basal ganglia in both normal and injured states (MPTP lesion) will provide valuable information in the development of new therapeutic strategies for neurodegenerative diseases such as Parkinson's disease, traumatic brain injury, and normal aging. Because 40–50% of the nigrostriatal dopaminergic neurons still remain in autopsied specimens of individuals affected with idiopathic Parkinson's disease, therapeutic strategies may be targeted toward restoring the functional capacity of these surviving neurons (Bernheimer et al., 1973; Pakkenberg et al., 1991). Potential strategies may include 1) increasing TH gene expression through modulating transcription factors, 2) increasing TH activity through elevated kinase activity, and 3) increasing neuronal sprouting and branching through neurotrophic factors. In addition to affecting TH activity, the posttranslational modifications of TH may influence its stability, translocation rate, and terminal localization (Jarrott and Geffen, 1972). Finally, understanding the intrinsic plasticity of the basal ganglia has important implications for the role of stem cell technology in Parkinson's disease as a modulator of neuroplasticity as well as an alternative source of dopamine.

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Exercise-Induced Behavioral Recovery and Neuroplasticity in the 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine-Lesioned Mouse Basal Ganglia

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Physical activity has been shown to be neuroprotective in lesions affecting the basal ganglia. Using a treadmill exercise paradigm, we investigated the effect of exercise on neurorestoration. The 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned mouse model provides a means to investigate the effect of exercise on neurorestoration because 30–40% of nigrostriatal dopaminergic neurons survive MPTP lesioning and may provide a template for neurorestoration to occur. MPTP-lesioned C57 BL/6J mice were administered MPTP (four injections of 20 mg/kg free-base, 2 hr apart) or saline and divided into the following groups: (1) saline; (2) saline + exercise; (3) MPTP; and (4) MPTP + exercise. Mice in exercise groups were run on a motorized treadmill for 30 days starting 4 days after MPTP lesioning (a period after which MPTP-induced cell death is complete). Initially, MPTP-lesioned + exercise mice ran at slower speeds for a shorter amount of time compared to saline + exercise mice. Both velocity and endurance improved in the MPTP + exercise group to near normal levels over the 30-day exercise period. The expression of proteins and genes involved in basal ganglia function including the dopamine transporter (DAT), tyrosine hydroxylase (TH), and the dopamine D1 and D2 receptors, as well as alterations on glutamate immunolabeling were determined. Exercise resulted in a significant downregulation of striatal DAT in the MPTP + exercise compared to MPTP nonexercised mice and to a lesser extent in the saline + exercised mice compared to their no-exercise counterparts. There was no significant difference in TH protein levels between MPTP and MPTP + exercise groups at the end of the study. The expression of striatal dopamine D1 and D2 receptor mRNA transcript was suppressed in the saline + exercise group; however, dopamine D2 transcript expression was increased in the MPTP + exercise mice. Immunoelectron microscopy indicated that treadmill exercise reversed the lesioned-induced increase in nerve terminal glutamate immunolabeling seen after MPTP administration. Our data demonstrates that exercise promotes behavioral recovery in the injured brain by modulating genes and proteins important to basal ganglia function.

Key words: tyrosine hydroxylase; dopamine transporter; dopamine receptors; glutamate; MPTP; Parkinson's disease

It has been established that neuroplasticity, as characterized by neurogenesis, synaptogenesis, and molecular adaptations, exists in the human nervous system. Animal models of brain injury have provided a means to both investigate and manipulate neuroplasticity. A heightened area of interest is the role that exercise plays in facilitating neuroplasticity in either the noninjured or injured brain (Fisher et al., 2001). Studies employing a variety of animal models of injury have shown that exercise can promote neuroplasticity and behavioral recovery in the hippocampus, cortex, and spinal cord (Kempermann et al., 2000). In

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rodent models of basal ganglia injury, exercise has been shown to be neuroprotective (Tillerson et al., 2001, 2003; Tillerson and Miller, 2002). By restraining the unimpaired limb immediately after injury and forcing use of the impaired upper limb, behavioral and neurochemical sparing were demonstrated in the 6-hydroxydopamine (6-OHDA)-lesioned rat. This suggested that forced use of the impaired limb protected dopaminergic cells from the neurotoxic effects of 6-OHDA. In addition, Tillerson et al. (2003) reported behavioral improvement after treadmill exercise in two rodent models of basal ganglia injury (the 6-OHDA rat and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [MPTP]-lesioned mouse) (Tillerson et al., 2003). Immediate exposure to treadmill training within 12 hr of injury was associated with attenuation of dopamine loss. The investigators concluded that exercise might work largely through neuroprotective mechanisms because exercise was started within 12 hr of lesioning, and MPTP and 6-OHDA may take several days to complete cell death (Sauer and Oertel, 1994; Jackson-Lewis et al., 1995). The implication of these neuroprotective studies is that exercise may be helpful in delaying or preventing Parkinson's disease in healthy individuals (Sasco et al., 1992).

The interest in our laboratory is to investigate the role of exercise in promoting repair of the injured basal ganglia. We define this as neurorestoration, which is the capacity of surviving dopaminergic neurons to adapt after injury with potential behavioral benefits. The MPTP-lesioned mouse model of basal ganglia injury provides a means to investigate neurorestoration because 30–40% of the substantia nigra pars compacta (SNpc) dopaminergic neurons survive the lesioning regimen. Despite a 90% loss of striatal dopamine, these mice display robust and reproducible return of striatal dopamine, tyrosine hydroxylase (TH) protein, and dopamine transporter (DAT) protein 2–3 months after MPTP lesioning. This suggests that surviving nigrostriatal dopaminergic neurons provide a template for neurorestoration and would therefore provide a means to investigate the effect of exercise on facilitating neurorestoration. In addition, MPTP-lesioned mice manifest motor behavioral deficits that can be monitored throughout the recovery process (Tillerson et al., 2003). The purpose of our study was to investigate the effect of treadmill exercise on neurorestoration using the MPTP-lesioned mouse model of basal ganglia injury by introducing exercise 4 days after MPTP lesioning, a time point well after cell death is complete. Because TH, DAT, dopamine (D1 and D2) receptors and glutamate storage have been shown to be altered in the MPTP-lesioned mouse model (Jakowec et al., 2004), we chose to examine these same parameters in our MPTP exercise paradigm. Exercise was continued for 30 days to parallel the 30-day recovery period when TH is beginning to return.

MATERIALS AND METHODS

Housing and Acclimation of Mice

Young adult (8–10 weeks old) male C57BL/6J mice supplied from Jackson Laboratory (Bar Harbor, ME) were used for this study. There were four treatment groups: (1) saline injected; (2) saline + exercise; (3) MPTP lesioned; and (4) MPTP lesioned + exercise. Three cohorts of mice, consisting of four groups of 10 mice/group were used (total n + 120 mice). Animals were housed six to a cage and acclimated to a 12-hr shift in light/dark cycle so that exercise occurred during the animals' normal wake period.

MPTP Lesioning

MPTP (Sigma, St. Louis, MO) was administered in a series of four intraperitoneal injections of 20 mg/kg (free-base) at 2-hr intervals for a total administration of 80 mg/kg. This regimen leads to a 60–70% loss of nigrostriatal neurons (as determined by unbiased stereologic techniques for both TH staining and Nissl substance in our laboratory) and an 80–90% depletion of striatal dopamine levels (Jackson-Lewis et al., 1995). Using this regimen, nigrostriatal cell loss is complete by Day 3 after MPTP administration and persists beyond 30 days post-lesioning as determined by either unbiased stereologic counting techniques (Petzinger et al., in preparation) or section-sampling techniques (Jackson-Lewis et al., 1995).

Selection of Mice and Exercise Protocol

Figure 1A outlines the experimental design of animal groups. Before MPTP lesioning, a baseline treadmill running assessment was conducted to insure that all animals performed similarly on the treadmill task before MPTP lesioning. Forty animals that could maintain a forward position on the 2.5-m treadmill belt for 5 min at 10 m/min were assigned randomly to the four groups. A non-noxious stimulus (metal-beaded curtain) was used as a tactile incentive to prevent animals from drifting back on the treadmill. Consequently, shock-plate incentive was not used and stress related to the activity was minimized. Exercise was initiated 4 days after saline or MPTP lesioning. For exercise training, a motorized, 10-lane rodent treadmill (Fig. 1B) was used at incremental speeds to a goal treadmill speed between 20.5–23.0 m/min (Fukai et al., 2000). All 10 mice from each of the two exercise groups (MPTP + exercise and saline + exercise) were run at the same time in the 10-lane treadmill (see Fig. 1B). Exercise duration was incrementally increased to reach the goal duration of 2 × 30 min/day (60 min), 5 days/week (with a 2-min warmup period) for a total of 30 days of exercise. Treadmill speed for each group was increased when all 10 mice within each group maintained a forward position on the 2.5-m treadmill belt for 75% of the running period. When all 10 mice within each of the two running groups maintained a forward position 100% of the time, duration for that group was increased. Mice were exercised as a group of 10 for two 30-min sessions (total 1 hr) per day with a 30-min rest period between sessions. To control for any non-exercise effects of treadmill running (handling, novel environment, noise, and vibration) nonexercised groups were placed on the top of the treadmill apparatus for a time period equivalent to

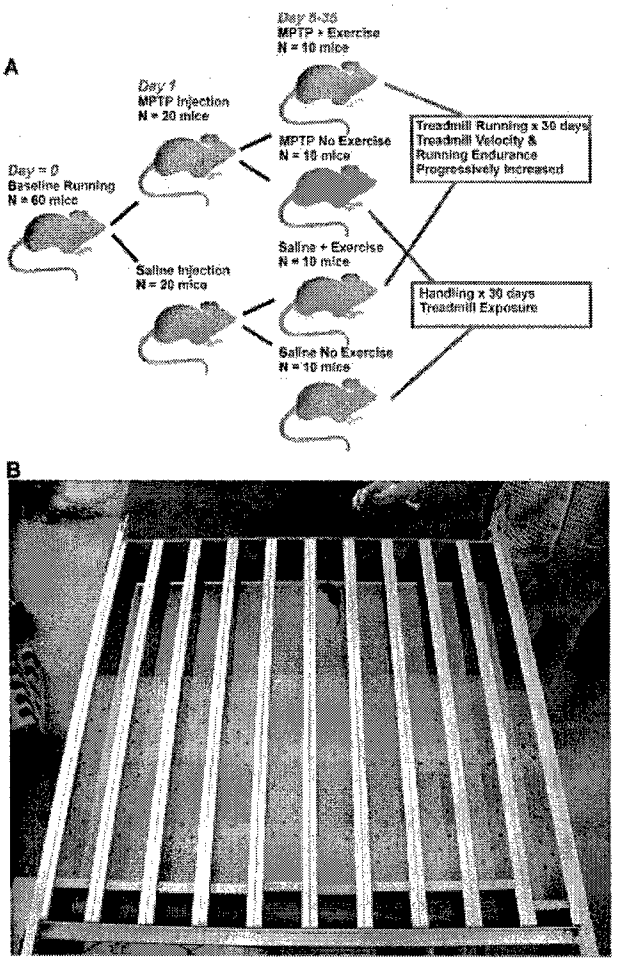

Fig. 1. **A:** Summary of experimental design with group assignment and exercise protocol. **B:** The 10-lane motorized treadmill used for exercising the mice.

exercise training (Fukai et al., 2000; Kojda et al., 2001). At the end of the 30-day running period, all animals from the four groups (exercise and non-exercise, with and without MPTP) were run to compare running speed capability. Initial treadmill velocity was set at the same speed at which initial pre-exercise baseline running capability was determined (i.e., 10 m/min). Maximum velocity for each group was defined as the velocity at which the mice, as a group of 10, could maintain a forward position on the treadmill for 75% of a 5-min running trial.

Collection of Brain Tissue

Brain tissue was collected at 4 days post-MPTP lesioning to examine the reduction in the degree of DAT and TH immunoreactivity. Brain tissue was collected from all groups at the conclusion of the 30 days of treadmill exercise, (35 days post-MPTP lesioning). Tissue for immunohistochemical analysis was fixed by transcardial perfusion with 50 ml of ice-cold saline followed by 50 ml of 4% paraformaldehyde/phosphate-buffered saline (PFA/PBS) pH 7.2. Brains were removed, post-fixed in 4% PFA/PBS for 48 hr, cryoprotected in 20% sucrose for 24 hr, and then quickly frozen in isopentane on dry ice. Tissues for Western immunoblotting and in situ hybridization were harvested fresh after cervical dislocation. All procedures used in these studies adhered to the guidelines of the Institutional Animal Care and Use Committee (IACUC) and the National Institutes of Health. To maximize the utilization of brain tissues each technique involving either fresh tissues (for Western immunoblotting), quick frozen tissues (for in situ hybridization histochemistry), or perfusion fixed (for immunoelectron microscopy or immunohistochemistry) consisted of at least 6 to as many as 10 mice from each group in a single experimental cohort. This assured a large enough n to detect changes within each experimental design.

Immunohistochemistry

Fixed tissue from at least six mice from each group was cut at 30-μm thickness, placed in phosphate buffer, and used immediately for immunohistochemistry. Commercially available antibodies included rabbit polyclonal anti-TH (Chemicon, Temecula, CA), and mouse monoclonal anti-DAT (Chemicon). Tissue sections were washed in Tris-buffered saline (TBS; 50 mM Tris pH 7.4 and 0.9% NaCl) and exposed to antibody (1:1,000) for 48 hr at 4°C. Sections were washed in TBS, and exposed to horseradish peroxidase (HRP)-conjugated secondary antibody using the ABC Elite kit (Vector Labs, Burlingame, CA). Antibody staining was visualized by development in DAB/H_2O_2. To ensure that differences in staining intensity were due to differences in antigen expression, multiple sections from each of the different treatment groups were handled concurrently in identical staining conditions. Control experiments excluding either primary antibody or secondary antibody were also carried out to verify staining specificity. Determination of the relative expression of TH immunoreactivity (ir) and DAT-ir in the striatum using immunohistochemistry from different mouse groups was based on published validity studies (Burke et al., 1990). For image analysis, three or four animals per treatment group and 10–12 sections per animal, spanning the midstriatum rostral to the anterior commissure (Bregma 0.25–1.25 mm) were used. Striatal images were captured at low magnification and digitized. The relative optical density (OD) (expressed as arbitrary units within the linear range of detection) of the dorsal lateral striatum was determined by subtracting the relative optical density of the corpus callosum as background. To ensure that the gray values represented an OD within the nonsaturated range of the image analysis, a Kodak Photographic step tablet (density rage to 255 OD units) captured by the CCD camera was used. Maximal tissue immunostaining relative OD units did not exceed the relative OD units of the tablet.

Western Immunoblotting

Tissue for Western blot analysis was dissected from the mid-striatum (a 3-mm thick section between Bregma 0.00–1.50) of at least six mice from each group and homogenized in buffer (25 mM Tris pH 7.4, 1 mM EDTA, 100 μM phenylmethylsulfonylfluoride [PMSF]). Protein concentration was determined by the BCA method (Pierce, Inc.). Proteins (10 μg) were

separated by polyacrylamide gel electrophoresis (PAGE) using the method of Laemmli (1970) and transferred to nitrocellulose filters by electroblotting in Towbin buffer (Towbin et al., 1979). Filters were blocked in TS-Blotto (50 mM Tris pH 7.4, 0.9% NaCl, 5% nonfat milk) for 1 hr, then exposed to primary antibody (1:2,000) in TS for 2 hr. Filters were then washed in TS, and exposed to secondary antibody in TS-Blotto for 1 hour. After a final wash in TS, antibody binding was visualized by chemiluminescence (Pierce, Inc.) and apposing filters to film (Hyperfilm ECL; Amersham) and processed in X-OMAT developer. Images were scanned and the relative OD of bands (expressed as arbitrary units within the linear range of film) was determined using Bioquant Nova Prime, a computer-assisted image analysis program (Bioquant Imaging, Nashville, TN).

Electron Microscopy/Immunocytochemistry

Electron microscopic immunolabeling for glutamate was carried out on mice from the saline group (n + 6), saline + exercise group (n + 10), MPTP group (n + 7), and MPTP + exercise group (n + 9). Anesthetized mice were perfused transcardially with 6 ml of heparin (1,000 U/ml) in HEPES buffer (pH 7.3) followed by 50 ml of 2.5% glutaraldehyde/0.5% paraformaldehyde in HEPES (pH 7.3) containing 0.1% picric acid. The brain was removed and post-fixed overnight at 4°C. Vibratome sections (200-mm thick) were cut in the coronal plane through the striatum and the dorsal hippocampus. A 2 × 2 mm^2 piece of the dorsolateral striatum (site of the major input of the corticostriatal pathway) and the CA1 subregion of the hippocampus (used as a control area to look for nonspecific effects of exercise), were dissected, washed in HEPES buffer, incubated at room temperature in the dark in aqueous 1% osmium tetroxide/1.5% potassium ferricyanide, washed in deionized water and en block stained with aqueous 0.5% uranyl acetate at room temperature for 30 min. The tissue was dehydrated, embedded in Embed 812/Spurr's (EMS; Fort Washington, PA) and sections were cut and stained. Post-embedding immunogold electron microscopy was carried out according to a modified method of Phend (Phend et al., 1992; Tillerson et al., 2003). Thin sections (light gold interface color) were cut and placed on 200-mesh nickel coated grids double coated previously with a solution from a Coat-Quick "G" pen (Kiyota International, Elk Grove, IL.), air dried for several hours, and washed for 5 min in TBS with Triton X-100 (TBST; 0.05 M Tris, pH 7.6, 0.9% NaCl, and 0.1% Triton X-100). The grids were transferred to the primary antibody solution and incubated overnight in a moist chamber. The glutamate antibody (non-affinity purified, rabbit polyclonal; Sigma, St. Louis, MO), as characterized previously by Hepler et al. (1988), was diluted 1:400,000 in TBST 7.6. Aspartate (1 mM) was added to the glutamate antibody mixture 24 hr before incubation with the thin-sectioned tissue to prevent any cross-reactivity with aspartate within the tissue. The grids were incubated for 1.5 hr at room temperature in goat anti-rabbit IgG conjugated to 10-nanometer gold (diluted 1:50 in TBST 8.2; Amersham). Photographs (10/animal) were taken randomly throughout the section containing the caudate nucleus or the CA1 region of the hippocampus (1 section/grid, one photograph per grid square) at a final magnification of 40,000× within the area of the neuropil (location of the greatest number of synapses) by an individual blinded to the particular experimental group and then captured on the computer using an AMT (2K × 2K) digital camera (Danvers, MA). The number of gold particles per nerve terminal associated with an asymmetrical (glutamate) synaptic contact and the area of the nerve terminal was determined using Image Pro Plus imaging software (Media Cybernetics, Tacoma, WA). The gold particles contacting the synaptic vesicles within the nerve terminal were counted and considered part of the vesicular or neurotransmitter pool by previously determined methods (Meshul et al., 1998; Tillerson et al., 2003). The specificity of immunolabeling for the glutamate antibody was established by incubating the antibody overnight with 3 mM glutamate. Preabsorption of the glutamate antibody resulted in a total lack of tissue labeling. The density of glutamate immunolabeling within the mitochondria associated with the presynaptic terminal was determined also as a means of investigating changes in the presynaptic metabolic pool of glutamate. The mean density of gold particles/μm^2 ± SEM) was determined within each treatment group. Stereologic analysis was not carried out for the nerve terminal glutamate immunolabeling study, because synapse density was not being determined, only the density of gold particles per identified nerve terminal making an asymmetrical synaptic contact.

In Situ Hybridization

Brains for in situ hybridization were removed quickly and frozen in isopentane on dry ice. Sections were cut 14-μm thick on a Jung 1850 cryostat (Leica, Inc.) and thaw mounted onto poly-L-lysine-coated microscope slides, dried on a 55°C slide warmer, and fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS), pH 7.2. Sections were dehydrated in successive ethanol washes (30, 60, 80, 95, and 100%), deprotonated in triethanolamine/acetic anhydride, delipidated in chloroform, and dehydrated in ethanol. Slides containing tissue sections were exposed to hybridization buffer containing 4× standard sodium citrate (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 50% formamide, 1× Denhardt's Solution, 250 μg/ml tRNA, 500 μg/ml salmon sperm DNA, and 10% dextran sulfate with 1 × 10^6 cpm of probe. Sections were cover-slipped and incubated overnight at 44°C. Tissue sections labeled with ribonucleotide probes were washed first in 2× SSC/50% formamide/0.1% β-mercaptoethanol at 37°C for 30 min, then 20 μg/ml RNase in 0.5 M NaCl/10 mM Tris pH7.4 at 37°C for 30 min, followed by 2× SSC/50% formamide/0.1% β-mercaptoethanol at 60°C for 1 hr, 0.1× SSC/0.1% β-mercaptoethanol at 65°C for 1 hour, and finally rinsed in ethanol before air drying. Slides were placed against high-resolution film (Hyperfilm B-max; Amersham) with radioactive standards (Amersham, Inc.). Selected slides were dipped in NTB-2 (Kodak) photographic emulsion, developed in D-19 developer and counter stained with eosin. To minimize potential sources of variation between different experiments, slides that were to be compared were processed in the same experiment using identical hybridization cocktail, probe concentration, probe preparation, wash regimen, and film exposure. The computerized image analysis program Bioquant was used to

determine the number of emulsion grains above specific anatomic regions within the substantia nigra pars compacta.

Statistical Analysis

Linear regression was carried out to compare the rate of change in velocity and endurance of treadmill running between the two groups. Treatment groups were compared using one-way analysis of variance (ANOVA), followed by the Fisher post hoc test for comparison of multiple means for the following measures: DAT, TH, dopamine D1 and D2 receptors, and glutamate immunogold labeling. All analyses were carried out with SPSS software. Statistical significance was accepted at $P < 0.05$.

RESULTS

Exercise-Induced Changes in Behavior

Figure 1 outlines the four animal groups used in this study. Two groups (saline + exercise and MPTP + exercise) were subjected to treadmill exercise for 30 days and changes in running duration and velocity were measured. Duration increased over the 30-day exercise period for both groups (see Fig. 2A). Both the saline + exercise and MPTP + exercise groups were capable initially of running for a duration of 30 min on Day 1, which increased to a maximal duration of 60 min. The saline + exercise group reached maximal duration by Day 12, however, whereas the MPTP + exercise group did not reach maximal duration until Day 26. As such, we compared the rate of increase in exercise duration over the first 12 days of running. The rate of change of duration for the saline + exercise group was significantly greater than that seen in the MPTP + exercise group ($P < 0.05$).

Similar to duration, running velocity increased in both the saline + exercise and MPTP + exercise groups over the 30-day period (see Fig. 2B). The saline group ran at a velocity of 13.3 m/min at Day 1 and increased to 23.0 m/min by Day 30. The MPTP group ran at a velocity of 6.3 m/min at day 1 and increased to 21.7 m/min by day 30. There was a significant difference in velocity at Day 1 between the two groups (saline + exercise, 13.3 m/min; MPTP + exercise

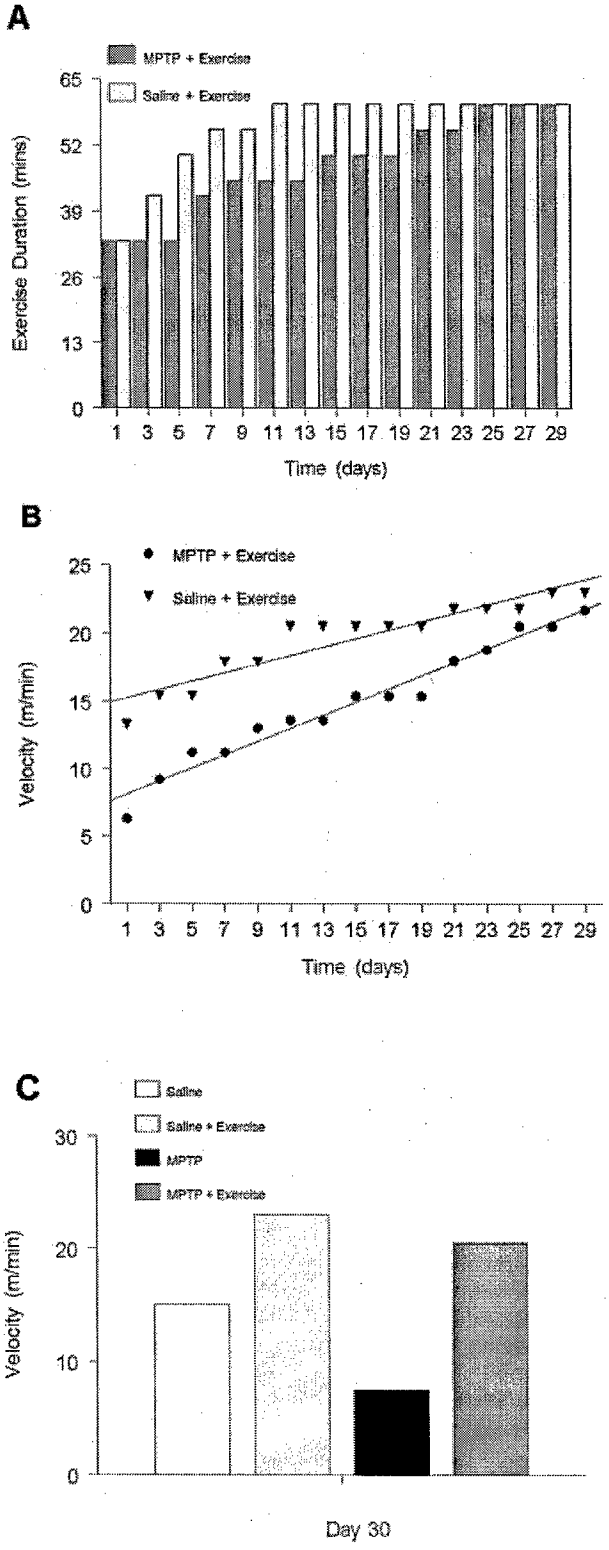

Fig. 2. Exercise-induced changes in behavior. **A:** Change in running duration over the 30-day running period for the saline + exercise group (gray bars) and MPTP + exercise group (black bars). The bars represent the performance of all 10 mice/group running at the same time. The increase over days of running reflects that all 10 mice met the criteria for increasing running duration. No statistical analysis was carried out because each bar represents all 10 mice/group as a single data point. **B:** Change in running velocity (in m/min) over the 30-day running period for the saline + exercise group (triangles) and MPTP + exercise group (circles). Symbols represent the performance of all 10 mice in each running group; increase over days of running reflects that all 10 mice met the criteria for increasing running velocity. **C:** Compares running velocity between the four groups (saline, white bar; MPTP, light gray bar; saline + exercise, black bar; and MPTP + exercise, dark gray bar) at the conclusion of the running program on Day 30. The bars represent performance of all 10 mice/group from the four groups running at the same time.

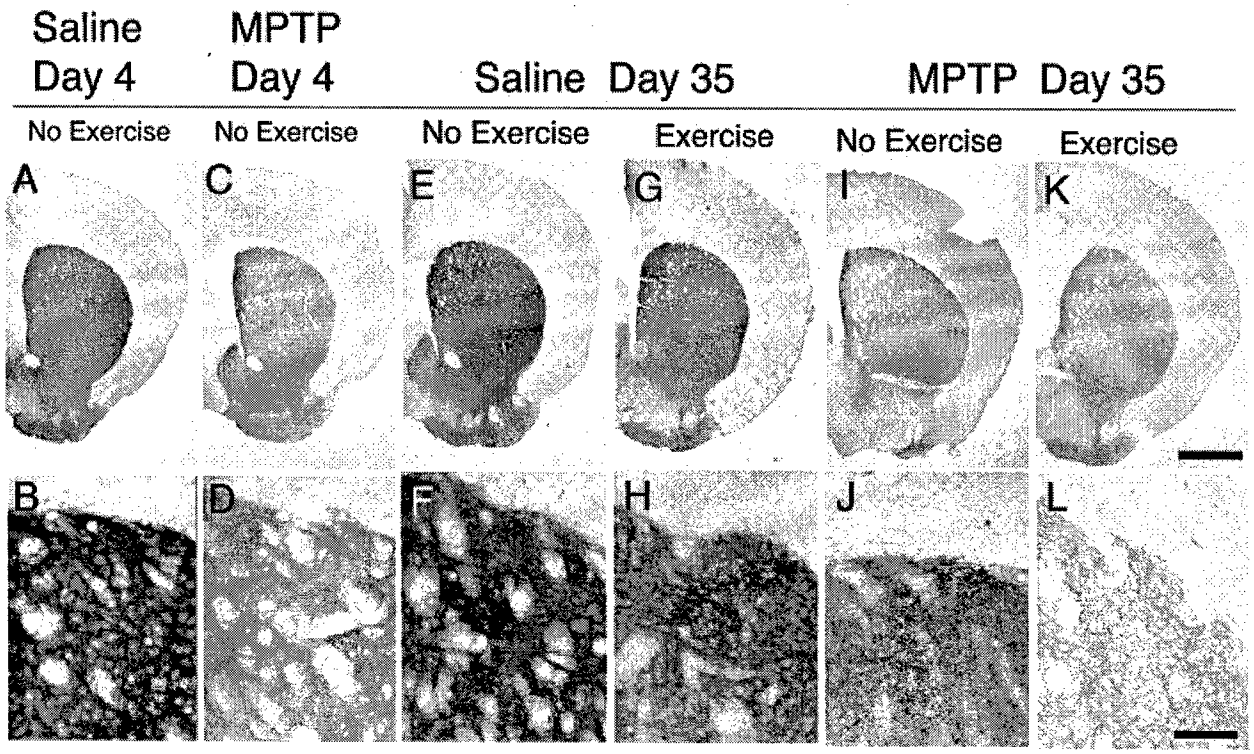

Fig. 3. Exercise-induced changes in striatal dopamine transporter immunolabeling. The relative expression of striatal dopamine transporter protein (DAT) after MPTP lesioning and between different treatment groups was determined using immunohistochemistry. The upper panels show low-magnification (10×) images of coronal sections at the level of the midstriatum stained with an antibody against DAT protein. The lower panels show high-magnification (400×) images from a region corresponding to the dorsal lateral striatum from respective sections in the upper panels. MPTP lesioning leads to a reduction in DAT-ir (comparing saline in **A** and **B** and MPTP in **C** and **D** at Day 4). MPTP + exercise showed reduced DAT-ir (**K** and **L**) compared to MPTP without exercise (**I** and **J**). Saline + exercise (**G** and **H**) showed a slight reduction in DAT-ir compared to saline alone (**E** and **F**). Scale bar + 0.5 mm (in K, for upper panels); 50 μm (in H, for lower panels).

group, 6.3 m/min; 7.0 m/min difference between groups at Day 1; $P < 0.0001$). This difference reduced to 1.3 m/min by Day 30 (Fig. 2B). The change in velocity over days of running resulted in a significantly different rate of change between the two groups ($P < 0.0001$). The MPTP + exercise group increased treadmill velocity by 5 m/min per day compared to 3.1 m/min per day for the saline + exercise group (Fig. 2B).

At the end of the 30-day running period, all animals from the four groups (saline, saline + exercise, MPTP, and MPTP + exercise) were tested on the treadmill to compare running speed capability (Fig. 2C). The MPTP and MPTP + exercise groups had a treadmill velocity of 7.5 and 21.7 m/min, respectively. Interestingly, the running velocity of the MPTP group at 35 days post-MPTP lesioning (7.5 m/min) was similar to the MPTP + exercise group at Day 1 (6.3 m/min) of their treadmill exercise program. Taken together, these findings indicate that there was no spontaneous increase in running velocity in the MPTP nonexercised group. The intensity of our training regimen was substantiated by the fact that there was an effect of exercise in the saline + exercise group compared to the saline nonexercised group. The comparison of running speed capability (Fig. 2C) at the end of the exercise program demonstrated differences in running velocity between the saline groups (saline + exercise, 23 m/min; saline nonexercised, 15 m/min).

Exercise-Induced Changes in Striatal Dopamine Transporter Protein

Analysis of the pattern of expression of DAT protein in the midstriatum (Bregma level +1.00) in both the saline (Fig. 3A,B) and MPTP groups (Fig. 3C,D) at Day 4 showed a significant reduction in DAT-ir due to MPTP lesioning. After the exercise regimen (Day 35 post-lesioning) the saline group showed the highest degree of striatal DAT-immunoreactivity (DAT-ir) compared to that in all other groups (Fig. 3E,F). Interestingly, the saline + exercise group had reduced DAT-ir compared to the saline group, which suggests that exercise itself reduced DAT-ir (compare Fig. 3E,F with Fig. 3G,H). This reduction was shown to be significant (saline, 100.0 ± 3.1%; saline + exercise, 84.3 ± 2.8%; $P < 0.006$) (see Fig. 4). In addition, the MPTP + exercise group demonstrated sig-

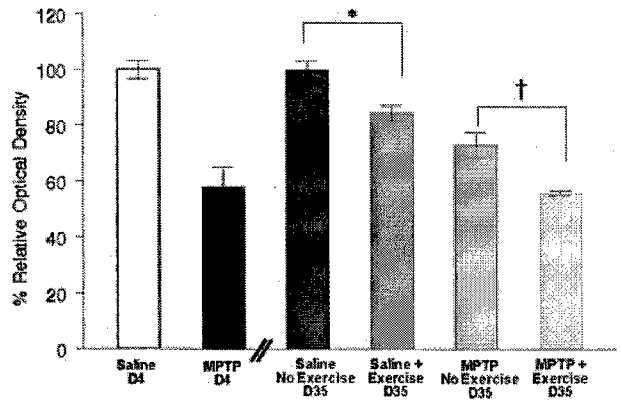

Fig. 4. Analysis of exercise-induced expression of dopamine transporter immunolabeling: The relative striatal DAT-ir was determined by measuring the relative optical density of the dorsal lateral quadrant (in at least 12 sections) correcting for staining background within sections by measuring the relative OD of the corpus callosum. This data represents relative staining for representative sections from the saline and MPTP groups at Day 4 post-MPTP lesioning and the four treatment groups collected after completion of the exercise program at post-MPTP lesioning Day 35. For comparisons, the Day 4 saline group was arbitrarily set as 100% (mean ± SEM, 100 ± 3.1%) and the relative optical densities of all other groups were normalized against it. Comparison of DAT-ir at Day 4 post-MPTP lesioning (58.0 ± 7.0%) showed a significant reduction in DAT-ir. The asterisks and cross indicate statistically significant differences between the saline and saline + exercise and the MPTP and MPTP + exercise groups, respectively. This reduction was significant (saline, 100.0 ± 3.1%; saline + exercise, 84.3 ± 2.8%; $P < 0.006$). In addition, the MPTP + exercise group demonstrated significantly reduced DAT-ir compared to the MPTP group (MPTP, 73.3 ± 4.5%; MPTP + exercise, 56.0 ± 0.7%; $P < 0.028$).

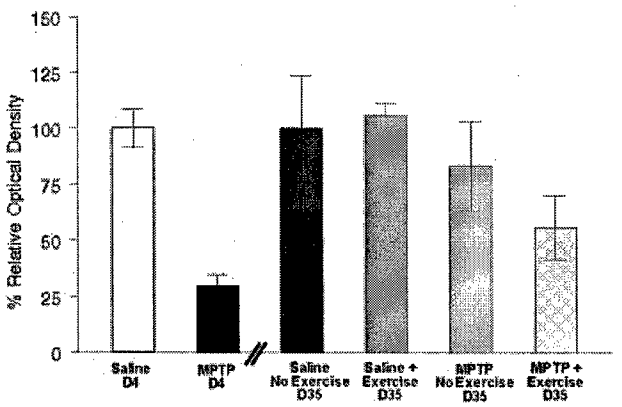

Fig. 5. Analysis of the relative striatal TH-protein expression by Western immunoblotting. The relative optical density measurement of the saline group at Day 4 post-lesioning was set arbitrarily at 100 ± 3.1% for comparison between different treatment groups. Analysis of TH-protein levels at 4 days after MPTP lesioning showed a significant reduction (30.0 ± 4.5%) compared to that in saline controls. There were no significant differences between groups in TH-ir at 35 days post-MPTP lesioning (saline, 100.0 ± 23.8%; saline + exercise, 105.8 ± 5.7%; MPTP, 83.3 ± 20.0%; MPTP + exercise, 56.0 ± 14.3%; $P + 3.72$). These data, however, indicate a small, nonsignificant reduction in the relative density of TH-ir in the MPTP groups compared to that in the saline groups. In addition, TH-ir was reduced slightly in the MPTP + exercise group compared to MPTP alone.

nificantly reduced DAT-ir compared to the MPTP group (MPTP, 73.3 ± 4.5%; MPTP + exercise, 56.0 ± 6.3%; $P < 0.028$) (see Fig. 4 and compare Fig. 3I,J with Fig. 3K,L).

Exercise-Induced Changes in Tyrosine Hydroxylase Striatal Protein

Western immunoblotting with an antibody recognizing TH protein was carried out on striatal tissue at post-lesioning Day 4 and at the end of the exercise program (35 days after MPTP lesioning) (Fig. 5). Comparison of the Day 4 saline with the Day 4 MPTP lesioning showed a significant reduction in striatal TH protein due to MPTP lesioning (saline, 100 ± 3.0% compared to MPTP, 30.0 ± 4.5%). Comparison of the level of TH protein at the end of the running regimen (at Day 35) showed that there was no significant difference in TH immunoreactivity between any of the groups (saline, 100.0 ± 23.8%; saline + exercise, 105.8 ± 5.7%; MPTP, 83.3 ± 20.0%; MPTP + exercise, 56.0 ± 14.3%, $P +$ 3.72). Although not significant (35 days after MPTP lesioning), MPTP groups (MPTP and MPTP + exercise) showed a slight reduction in TH immunoreactivity compared to the saline groups. In addition, TH immunoreactivity was reduced slightly in the MPTP + exercise group compared to that in the MPTP group.

Exercise-Induced Changes in Dopamine D1 and D2 Receptors

In situ hybridization histochemistry with probes recognizing either the D1 or D2 subtype of dopamine receptor was carried out on dorsal striatal tissue of mice from all groups. Compared to the saline group, expression of D1 mRNA was reduced as a result of exercise, MPTP lesioning, or both (saline, 100.0 ± 6.9%; saline + exercise, 51.9 ± 3.9%; MPTP, 50.1 ± 5.9%; MPTP + exercise, 48.6 ± 4.2%; $P < 0.0001$) (Fig. 6A). Comparison of the MPTP with the MPTP + exercise group showed no difference in the level of dopamine D1 mRNA expression. The expression of dopamine D2 mRNA was also reduced as a result of either exercise (saline + exercise) or MPTP lesioning (MPTP group) compared to that in the saline group (saline, 100.0 ± 7.6%; saline + exercise, 58.5 ± 6.4%; MPTP, 50.1 ± 5.7%; $P < 0.002$) (Fig. 6B). The combination of MPTP lesioning and exercise (MPTP + exercise group), however, resulted in no difference in dopamine D2 mRNA expression when compared to that in the saline group, but was increased significantly when compared to that in the MPTP group (MPTP + exercise, 95.9 ± 9.4%; $P < 0.005$).

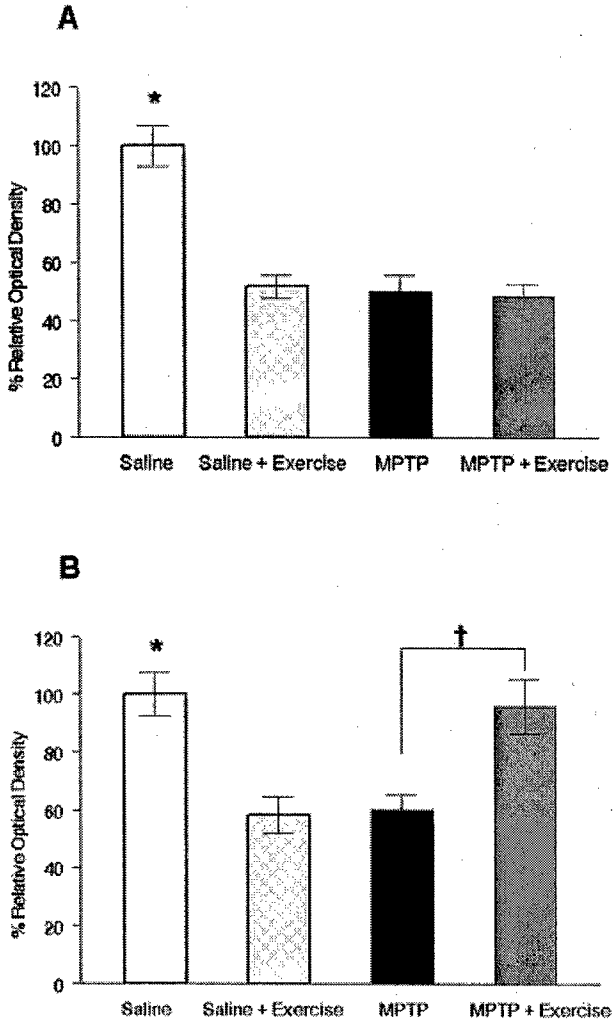

Fig. 6. Analysis of the relative striatal dopamine D1 and D2 receptor mRNA using in situ hybridization histochemistry. The relative optical density of autoradiographic grains above the dorsal striatum were determined from at least three mice from each treatment group using at least 12 sections/mouse. For comparison, the saline group was set arbitrarily at 100% and all other groups normalized against it for both dopamine receptors D1 and D2 mRNA. **A:** Compared to the saline group, expression of D1 mRNA was reduced significantly with exercise and MPTP lesioning (saline, 100.0 ± 6.9%; saline + exercise, 51.9 ± 3.9%; MPTP, 50.1 ± 5.7%; MPTP + exercise, 48.6 ± 4.2%; $P + 0.0001$). **B:** The expression of dopamine D2 mRNA was reduced as a result of either exercise (saline + exercise) or MPTP lesioning (MPTP group) compared to that in saline group (saline, 100.0 ± 7.6%; saline + exercise, 58.5 ± 6.4%; MPTP, 60.3 ± 5.3%; $P < 0.002$). The expression of dopamine D2 mRNA was increased significantly in the MPTP + exercise group when compared to that in the MPTP group (MPTP + exercise, 95.9 ± 9.4%; $P < 0.005$).

Exercise-Induced Changes in Nerve Terminal Glutamate Immunolabeling

Immunogold electron microscopy was used to determine the density of nerve terminal glutamate immunolabeling in mice from all groups at completion of the exercise program. Figure 7 shows representative images of asymmetrical (excitatory) synaptic contacts labeled for the neurotransmitter glutamate in mice from the saline group, saline + exercise group, MPTP group, and the MPTP + exercise group (Fig. 7A–D, respectively). There was a significant increase in the density of nerve terminal glutamate immunolabeling in the MPTP group compared to saline, (values are mean number of gold particles/μm^2 ± SEM: saline, 85.9 ± 3.6; MPTP, 135.3 ± 12.4; $P < 0.05$) (Fig. 8). Additionally, there was a significant decrease in the density of nerve terminal glutamate immunolabeling in the MPTP + exercise group compared to the MPTP group (MPTP, 135.3 ± 12.4; MPTP + exercise, 105.3 ± 4.5; $P < 0.05$). This decrease reached levels that were similar to the saline groups (mean number of gold particles/μm^2 ± SEM: saline, 85.9 ± 3.6; saline + exercise, 102.6 ± 3.7; MPTP, 135.3 ± 12.4; MPTP + exercise, 105.3 ± 4.5).

To determine the specificity of the change in the density of glutamate immunolabeling within the nerve terminal, the density of labeling within the presynaptic mitochondrial pool was quantified. There was no difference between any of the experimental groups (data not shown). Additionally, there were no changes in nerve terminal area between groups (data not shown).

Glutamate immunolabeling of the CA1 in the hippocampus was analyzed to confirm that the observed differences between groups in glutamate immunolabeling were specific to the striatum. This brain area was chosen because it also receives a significant glutamatergic input and is associated with spatial learning as opposed to the dorsolateral striatum that is associated primarily with motor function. There were no differences in the density of nerve terminal glutamate immunolabeling between any of the groups as shown in Figure 8B (values are mean number of gold particles/μm^2 ± SEM; saline, 100.0 ± 5.7; saline + exercise, 101.7 ± 7.9; MPTP, 88.4 ± 4.6; MPTP + exercise, 103.2 ± 6.6; $P < 0.49$).

DISCUSSION

The purpose of this study was to examine the effect of exercise on restoration of surviving dopaminergic neurons after completion of MPTP-induced cell death. The MPTP lesioning regimen used in our studies involves a series of four injections of 20 mg/kg (free-base) leading to a 60–70% loss of nigrostriatal dopaminergic neurons and a 90–95% depletion of striatal dopamine (Jackson-Lewis et al., 1995). Using this regimen, nigrostriatal cell loss is complete by Day 3 after MPTP administration and shows no further decline 30 days post-lesioning as determined by either unbiased stereologic counting techniques (Petzinger et al., in preparation) or section-sampling techniques (Jackson-Lewis et al., 1995). Despite the extent of cell loss, MPTP-lesioned mice display robust and reproducible return of striatal function 2–3 months after injury (Ricaurte et al., 1986; Jakowec et al., 2003). The levels of TH and DAT immunoreactivity (TH-ir and DAT-ir) are de-

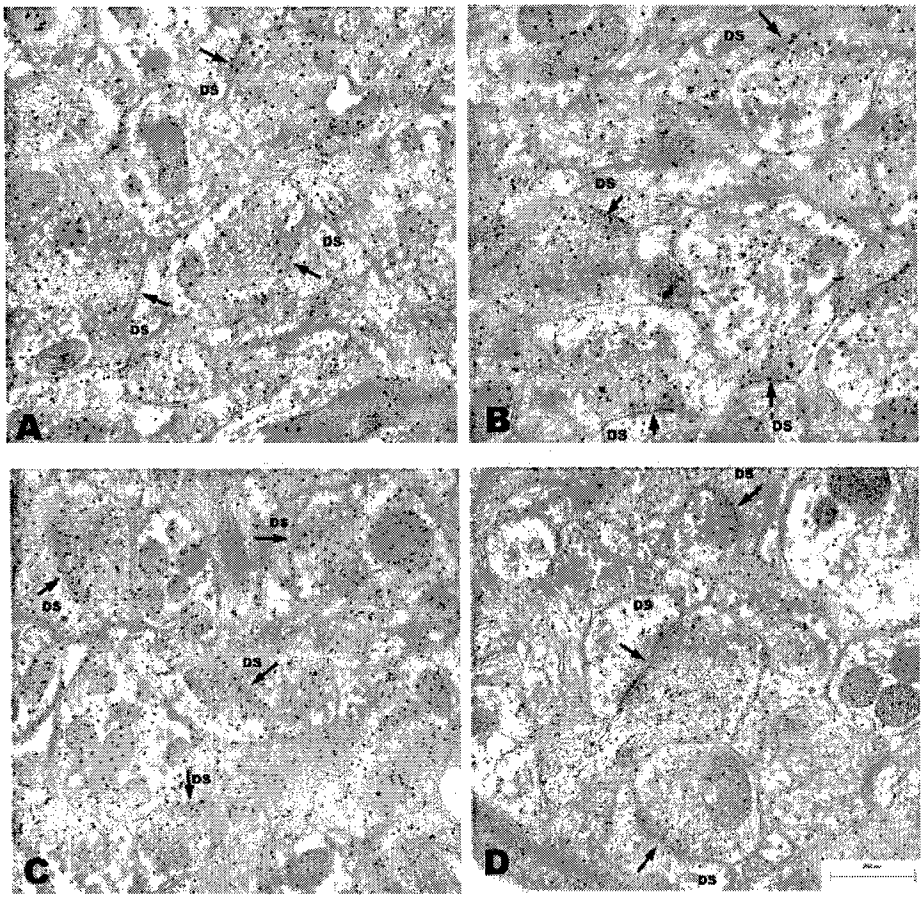

Fig. 7. Electron photomicrographs using the immunogold technique to localize an antibody against the neurotransmitter, glutamate, within the dorsolateral striatum. **A:** Saline group. Three nerve terminals are seen making an asymmetrical synaptic contact (arrows) with an underlying dendritic spine (DS). Within the nerve terminal are numerous 10-nm gold particles, indicating the location of the antibody. These gold particles are found overlying the round synaptic vesicles. **B:** Saline group that was exercised for 30 days, starting 4 days after the injection of saline. Note that the density of nerve terminal glutamate immunolabeling seems similar to that seen in the saline-treated group in A. **C:** MPTP-treated group was given an acute injection of the toxin (20 mg/kg × 4 injections every 2 hr) and then perfused with fixative 34 days later. Note the increase in the density of immunogold particles in all three nerve terminals compared to that observed in the saline group shown in A. **D:** MPTP-treated group that was exercised for 30 days, starting 4 days after the acute toxin treatment. Note that the density of glutamate immunogold labeling is similar to that seen in the saline-treated group in A. Scale bar + 0.25 μm.

creased to 30% of pre-MPTP-lesioned levels by Day 7 and return to 50–60% or more of pre-MPTP-lesioned levels within 30–60 days (Jakowec et al., 2003, 2004). These changes are part of molecular alterations underlying intrinsic neuroplasticity in this model (Jakowec et al., 2004) and are represented in this study by the MPTP nonexercised group. The saline group in this study served two purposes: (1) to show that the intensity of the exercise regimen was sufficient to induce a behavioral effect in non-lesioned controls; and (2) to compare the effect of exercise on the noninjured and injured brain.

Using a high-intensity (high velocity and duration) treadmill exercise paradigm in the MPTP-lesioned mouse, we have shown that exercise leads to behavioral recovery, specifically amelioration of initial deficits in running speed and duration compared to nonexercised MPTP-lesioned animals. Importantly, as would be expected by an effective training paradigm, the non-lesioned animals that exercised demonstrated enhanced performance compared to their non-lesioned, nonexercised counterparts. Specifically these behavioral differences between exercised and nonexercised mice consisted of the capability of the exercised saline mice to run at higher velocities after a 30-day treadmill-training program. We showed that MPTP-lesioned and non-lesioned mice could be forced to run at progressively faster speeds and longer durations and learn to associate a sensory stimulus with a behavioral response (i.e., maintaining a specific position on the treadmill). Over time, sensory feedback was no longer necessary for the animals to maintain a forward position, indicating that learning had occurred.

Although the MPTP-lesioned mouse displays subtle motor behavioral deficits that may not be evident under normal caging conditions, motor deficits become evident under specific task or environmental manipulations (Sedelis et al., 2001; Tillerson et al., 2002). Bradykinesia (slowness of movement) and fatigability (decreased endurance) were two behavioral deficits we observed initially in treadmill-exercised mice after MPTP lesioning. MPTP-lesioned mice were not capable of running at the same treadmill velocity as the saline + exercise mice in the first 18 days of running. By the end of the 30-day exercise program, the MPTP + exercise mice were running at a velocity near that of the saline + exercise group and greater than that of the saline (nonexercised) group (see Fig. 2C). The rate of change of velocity was greater in the

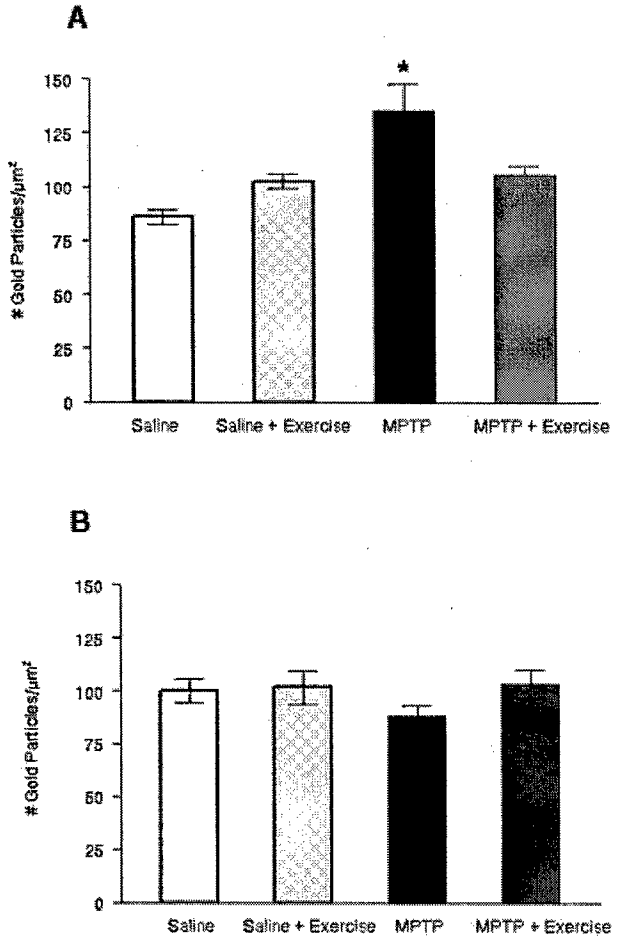

Fig. 8. Quantification of immunogold electron microscopy results. **A:** There was a significant increase in the density of synaptic glutamate immunolabeling within striatal nerve terminals making asymmetrical synaptic contacts after the acute administration of MPTP compared to the other treatment groups (mean number of gold particles/μm^2 ± SEM: saline, 85.9 ± 3.6; saline + exercise, 102.6 ± 3.7; MPTP, 135.3 ± 12.4; MPTP + exercise, 105.3 ± 4.5; $P < 0.05$). **B:** Using quantitative immunogold electron microscopy, there was no difference in the density of glutamate immunolabeling within nerve terminals making an asymmetrical synaptic contact within the CA1 region of the hippocampus between any of the treatment groups. *$P < 0.05$ compared to all the other groups as determined by the Fisher post-hoc test for comparison of multiple means.

injured animals compared to their nonlesioned exercised counterparts. This result is in accordance with exercise studies in cortically injured animals in which the effect of exercise is greater after injury. Injury may prime the system for adaptation perhaps through the induction of neurotrophic factors including brain-derived neurotrophic factor (BDNF) (Cotman and Berchtold, 2002; Gomez-Pinilla et al., 2002; Cohen et al., 2003).

Tillerson et al. (2003) also reported behavioral improvement after treadmill exercise in two rodent models of basal ganglia injury (the 6-OHDA rat and MPTP-lesioned mouse), which was associated with attenuation of dopamine loss (Tillerson et al., 2003). The investigators concluded that exercise might work largely through neuroprotective mechanisms because exercise was started within 12 hr of lesioning, and MPTP and 6-OHDA may take several days to complete cell death (Sauer and Oertel, 1994; Jackson-Lewis et al., 1995). Unlike the Tillerson et al. (2003) study, the focus of our study was to address the effect of high-intensity treadmill exercise on the neurorestoration of surviving neurons after MPTP lesioning. This was accomplished by: (1) initiating exercise 4 days after lesioning, a time period well after cell death is completed in this model; (2) continuing exercise over a 30-day period; and (3) progressively increasing treadmill velocity and duration over that period. Two additional differences between Tillerson et al. (2003) and the present study were the exercise parameters and the age of the animals. Our exercise parameters on young mice were of higher velocity, duration, and frequency and showed an effect on the saline + exercise group that was not seen in the Tillerson et al. (2003) study.

In addition to a behavioral effect, exercise resulted in decreased DAT-ir compared to the nonexercised groups. The effect of exercise on DAT-ir was even greater in the MPTP group. In the basal ganglia, the biosynthesis of dopamine is dependent on the enzyme tyrosine hydroxylase (TH) and the primary mechanism of clearance of dopamine from the extracellular space is through the dopamine transporter (DAT) (Gainetdinov et al., 2002; Mortensen and Amara, 2003). Several mechanisms have been shown to regulate DAT activity including: (1) gene and protein expression of transporter number; (2) phosphorylation activated through glutamate receptors such as the mGluR5 metabotropic receptor; and (3) internalization within endosomes mediated by dopamine (Perrone-Calano et al., 1996; Page et al., 2001). Alterations in DAT activity can influence the synaptic occupancy of dopamine. An intervention (such as exercise) that downregulates DAT-ir expression may therefore lead to behavioral improvement by increasing synaptic occupancy of dopamine. The downregulation of DAT-ir in our exercised animals could account for the superior running capabilities of both the MPTP and saline groups compared to the nonexercised groups. This interpretation is consistent with the findings of Meeusen et al. (1997) and others that report increased extracellular levels of dopamine with exercise. An alternative explanation for reduced DAT-ir in our exercised animals is the loss of nigrostriatal terminals where DAT normally resides. This does not seem likely because TH-ir, another marker of nigrostriatal terminal integrity, was not altered significantly and because exercised animals had superior running capability compared to their nonexercised counterparts, which would not be expected if cell death were ongoing. Studies are underway currently to investigate the possibility of cell death and terminal loss

including stereologic cell counting of substantia nigra pars compacta neurons and fiber density, respectively.

In saline animals, exercise suppressed dopamine D1 and D2 receptor mRNA levels. In the MPTP group, exercise seemed to have no effect on D1 but increased D2 mRNA levels. Of the dopamine receptor superfamily, D1 and D2 subtypes are the most prevalent in the striatum (REF). Activation of these receptors by dopamine leads to the release of neuropeptides from medium spiny neurons. Medium spiny neurons with D1 receptors express the neuropeptide preprotachykinin (PPT) and medium spiny neurons with D2 receptors express the neuropeptide preproenkephalin (PPE) (Gerfen, 2000). In the normal brain, D1 and D2 act synergistically and activation of both is required to elicit a motor response (Gerfen et al., 1995). In the lesioned basal ganglia, this synergy is lost and activation of either D1 or D2 may elicit a motor response. In addition, D2 activation alone in the injured state seems to elicit a more robust motor response that may be attributed to its heightened sensitivity after lesioning (LaHoste and Marshall, 1993). In our study, exercise seems to have a similar effect on both receptor subtypes in the saline animals that may reflect the synergy normally seen in the uninjured basal ganglia. This synergy, however, is lost in injury and affects each receptor subtype differently. The loss of synergy between the dopamine D1 and D2 receptors due to injury by MPTP is revealed in the context of exercise. One possible mechanism to explain the differential effect of exercise and injury on the dopamine receptors subtypes D1 and D2 may be through the action of glutamate. Glutamate has been shown to influence subtype-specific regulation of the dopamine receptors (see discussion below). The combination of the upregulation of D2 mRNA (leading to increased motor activity) along with the downregulation in DAT (leading to increased synaptic occupancy of dopamine) may explain the behavioral improvement seen in the MPTP + exercise mice. Studies are underway currently to localize the dopamine D2 receptor changes with exercise and to determine if D1 or D2 receptor antagonists or agonists affect the behavioral benefits of exercise.

Glutamate is the major excitatory neurotransmitter in the brain and plays an important role in motor behavior (Starr, 1995). Glutamate is stored within nerve terminals and upon release binds to a superfamily of receptors including the *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA), and kainic acid (KA) subtypes. In the striatum, the primary glutamatergic pathway is the corticostriatal input to the medium spiny neurons (Starr, 1995). One method for examining changes in glutamatergic neurotransmission is to measure alterations in glutamate storage within striatal nerve terminals using immunogold electron microscopy. We observed an increase in the density of nerve terminal glutamate immunolabeling in animals after MPTP lesioning. This increase was reversed by exercise to levels seen in the saline control groups. Additionally, this effect was specific to the lesioned dorsal-lateral striatum (an area associated primarily with motor function) because there was no alteration in CA1 glutamate terminals originating from either the Schaffer collaterals or from the contralateral hippocampus (an area associated primarily with learning and memory). In comparison to the MPTP-lesioned animals, no significant change in immunogold labeling was observed between the saline and saline + exercise groups. A change in glutamate terminal storage in the nonlesioned brain may require a higher intensity of exercise then used in the present studies (Meeusen et al., 1997).

Although glutamate levels were not measured in this study, Meshul et al. (2000) has shown an inverse relationship between terminal glutamate immunogold labeling and levels of glutamate within the synapse. An increase in the density of nerve terminal glutamate immunolabeling (as is seen with MPTP lesioning) may therefore reflect a decrease in the extracellular levels of striatal glutamate. Consequently, one hypothesis with respect to our results is that an effect of exercise in the MPTP-lesioned brain may be to increase the release of glutamate at the synapse, which that may alter dopamine receptor subtype expression or medium spiny neuron peptide expression (Cepeda et al., 1993; Cepeda and Levine, 1998; Liste et al., 1999). Using microdialysis in the 6-OHDA rat, Meeusen et al. (1997) showed an increase in extracellular glutamate with exercise (Meeusen et al., 1997; Bland et al., 1999).

Studies have shown that there are close interactions between glutamate and dopamine neurotransmission in mediating motor control (Starr and Starr, 1994; Starr, 1995; Starr et al., 1997). The striatal medium spiny neuron is thought to be the site for integrating these interactions. Exercise may either directly affect the medium spiny neuron or indirectly influence its afferents. For example, expression of the immediate early gene cFos (a marker of cell activation) and the neuropeptides preprotachykinin and preproenkephalin in medium spiny neurons are altered by exercise (Cepeda et al., 1993; Liste et al., 1999). Altered expression of these markers in medium spiny neuron activity can be blocked by either glutamate or dopamine receptor antagonists or through denervation. The present study has shown changes in both glutamate and dopamine systems. We do not yet know, however, if glutamate and dopamine changes are dependent or mutually exclusive of each other. To test the degree of dopamine–glutamate interactions with exercise, we are conducting additional studies with exercise in MPTP and saline mice administered either glutamate or dopamine antagonists. If the suppression of DAT-ir seen in our studies can be blocked by administration of a glutamate antagonist during exercise, it would support the hypothesis that glutamatergic neurotransmission is important in regulating exercise-induced changes in dopamine function. Furthermore, dopamine receptor-specific agonists and antagonists targeting either D1 or D2 will test whether the alterations in glutamate immunolabeling seen in our studies are dependent on dopamine neurotransmission.

In conclusion, exercise may be both neuroprotective and neurorestorative in the injured basal ganglia. It has been shown previously that initiating exercise at or during the time of neurotoxin-induced cell death is neuroprotective by attenuating striatal dopamine loss (Cohen et al., 2003; Tillerson et al., 2003). Our studies show that a high-intensity treadmill exercise paradigm initiated after the period of neurotoxin-induced cell death is neurorestorative as demonstrated through its beneficial effect on motor behavior. Alterations in both dopaminergic and glutamatergic neurotransmission in response to exercise may underlie the molecular mechanisms of this effect. The potential impact of this study is that exercise may not only play a role in prevention of Parkinson's disease but in restoring function in individuals who have been diagnosed with Parkinson's disease.

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Behavioral Motor Recovery in the MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)-Lesioned Squirrel Monkey (Saimiri sciureus) is Not Solely Dependent on Striatal Dopamine nor the Expression of Tyrosine Hydroxylase and Dopamine Transporter Proteins

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Abbreviated Title: Recovery in the MPTP Nonhuman Primate

Key words: basal ganglia, Parkinson's disease, nonhuman primate, neuroplasticity.

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ABSTRACT

The neurotoxicant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) provides an excellent opportunity to study repair and response to injury in the basal ganglia. Its administration to mammals leads to the destruction of nigrostriatal dopaminergic neurons and striatal dopamine depletion. In the squirrel monkey (*Saimiri sciureus*) MPTP-lesioning results in parkinsonian motor symptoms including bradykinesia, postural instability, and rigidity. Several months after MPTP-lesioning, squirrel monkeys recover motor behavior to a level indistinguishable from non-lesioned animals. The few studies examining behavioral recovery suggest an incomplete return of dopamine. Proposed compensatory mechanisms include (i) an inverse adjustment in dopamine turnover ratio; (ii) decreased expression of the dopamine transporter resulting in increased volume transmission; and (iii) sprouting of surviving dopaminergic neurons. For these studies we employed a lesioning regimen of 2 or 6 subcutaneous injections of MPTP at a concentration of 2.0 mg/kg (free-base) to generate monkeys with mild or severe motor deficits, respectively. Brain tissue was harvested at 6 weeks or 9 months after the last injection and analyzed for dopamine and its metabolites, and expression of tyrosine hydroxylase (the rate-limiting step in dopamine biosynthesis), and dopamine transporter (responsible for dopamine uptake). Finally, we examined the expression of dopamine- and cAMP-responsive protein phosphatase of 32 KDa (DARPP-32), an effector molecule involved in basal ganglia function. Our results showed that recovery processes are dynamic, and compensatory mechanisms associated with the dopaminergic system are not sufficient to explain motor recovery suggesting that non-dopaminergic systems, including glutamate, may play an important role in recovery.

INTRODUCTION

The neurotoxicant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) provides an excellent opportunity to study repair and the response to injury in the basal ganglia. MPTP is a meperidine derivative that can be administered systemically. MPTP, the pre-toxin form, crosses the blood brain barrier and is converted to the toxic form 1-phenyl-4-phenylpiperidium (MPP^+) by astrocytic monoamine oxidase B (Chiba et al. 1985). MPP^+ acts as a "false-substrate" for the dopamine transporter (DAT) and accumulates in dopaminergic neurons where it targets mitochondrial complex I leading to energy depletion and the formation of reactive oxygen species (Dauer and Przedborski 2003). This results in the selective destruction of nigrostriatal dopaminergic neurons and the depletion of the striatal neurotransmitter dopamine similar to that seen in Parkinson's disease (PD) (Jakowec and Petzinger 2004). Dopamine depletion results in behavioral motor deficits in both mice and nonhuman primates. Motor behavioral changes in mice tend to be subtle and require specific behavioral testing including treadmill, paw-reach, and rotorod balancing to become evident (Fisher et al. 2004; Sedelis et al. 2000; Sedelis et al. 2001; Tillerson et al. 2001). In nonhuman primates motor behavioral deficits resembling those seen in humans with PD, or drug addicts who self-administered MPTP are evident and include akinesia, bradykinesia, postural instability, freezing, and in some species a resting tremor (Burns et al. 1983; Langston et al. 1983; Langston et al. 1984).

MPTP has been administered to a variety of different nonhuman primates using a several different regimens. In our laboratory we utilize the squirrel monkey (*Saimiri sciureus*), a New World monkey. We have developed a lesioning regimen that consists of either a series of 2 or 6 injections of MPTP at a concentration of 2.0 mg/kg (free-base) with 2 weeks between injections. With this lesioning regimen we avoid high animal mortality often experienced with MPTP use. Behavioral analysis using a clinical rating scale (CRS) we specifically designed for the MPTP-lesioned squirrel monkey, documents either mild (2 injections) or severe (6 injections) parkinsonian motor behavior. Interestingly, over time animals display

motor recovery so that by 6 weeks post-MPTP, animals show partial motor recovery and by 9 months post-MPTP, both groups of monkeys show motor behavior that is indistinguishable from non-lesioned animals. Specifically, recovered animals no longer display akinesia or bradykinesia and show normal spontaneous movement (climbing and jumping), normal hand dexterity, and balance. In the few studies that have examined behavioral recovery, it has been seen that recovery takes place with incomplete return of dopamine and that the alterations in the dopaminergic system that account for recovery are mostly localized to the ventral striatum (Elsworth et al. 2000; Rose et al. 1989b; Rothblat and Schneider 1994; Schneider et al. 1998). It has been suggested that as compensation for the deficient return of dopamine that there is (i) an inverse adjustment in turnover rate such that a paucity of dopamine return would be accompanied by a tremendous turnover rate; (ii) a decreased expression of the dopamine transporter protein thereby increasing diffusion of dopamine from the ventral region; and (iii) sprouting of surviving dopaminergic neurons as indexed by the return of expression of TH protein. The goal of this study was to add to this important body of literature with respect to the mechanisms that account for recovery in our animals and to test whether the aforementioned compensations are specific features of the recovery. In addition, we examined if the underlying mechanisms changed over time by comparing early (6 weeks post-MPTP)- and late (9 months post-MPTP)-stage recovery animals. Finally, we hypothesized that if alterations in the dopaminergic system solely accounted for behavioral recovery then this would be reflected by a relatively normal expression of a downstream effector molecule of dopamine within the basal ganglia, such as dopamine- and cAMP-responsive protein phosphatase of 32 KDa (DARPP-32).

MATERIAL AND METHODS

Animals

A total of 24 young-adult male squirrel monkeys (*Saimiri sciureus*) weighing between 900 and 1200 grams each were used in these studies (Osage, St. Louis, MO). All procedures utilizing the non-human primate strictly followed guidelines set forth by the National Institutes for Health for the humane

treatment of animals in research and had the approval of the University of Southern California Institutional Animal Care and Use Committee (IACUC). Animals were housed individually in a home cage. Following quarantine, animals were acclimated to the facility for 30 days prior to behavioral analysis in their home cages.

MPTP-Lesioning

MPTP (Sigma Corp, St. Louis, MO; catalog M0896) was administered in a series of subcutaneous injections set 2 weeks apart at a concentration of 2.0 mg/kg (free-base) dissolved in sterile water and made up fresh from a new 100 mg bottle each time. One group of animals (n = 8) received a series of 2 injections (for a total of 4.0 mg/kg MPTP, free-base). Another group of animals (n = 8) received a series of 6 injections of MPTP (for a total of 12.0 mg/kg, free-base). A saline injected group (n = 8) acted as control.

Clinical Rating Scale for Motor Behavioral Analysis

Behavioral motor features were determined using a cage-side clinical rating scale (CRS) based on the Unified Parkinson's Disease Rating Scale (UPDRS) and modified for the squirrel monkey. Table 1 outlines the features of the clinical rating scale. The CRS consisted of 6 items including (1) spatial hypokinesia (movement around cage), (2) body bradykinesia, (3) manual dexterity in left arm, (4) manual dexterity in right arm, (5) balance, and (6) freezing. Each item has a score of 0 to 4 resulting in a maximum motor score deficit of 24 points. A CRS score of less than 4 points is within the range of normal. In addition, animals were assessed for alertness, cognition, and motivation, based on interactions with the CRS raters and the interest and ability of an animal to identify, track, and acquire food rewards. Two investigators, blinded to the animal and treatment group, assessed motor and cognitive features three times per week in the morning before feeding time. Baseline behavior was determined in the two week period before the first injection of MPTP or saline. Parkinsonian motor features using the CRS were

determined and cognitive changes noted starting two weeks after the last injection of MPTP or saline, and continued on a weekly basis until tissue harvesting at 6 weeks or 9 months. For statistical analysis, the weekly CRS scores for each animal were averaged. The weekly CRS of all animals within each group (n = 4/group) were averaged. To evaluate the performance on the clinical rating scale, a mixed model ANOVA was used. The between-subject factor is Group (saline, 2-time MPTP injected, and 6-time MPTP injected) and the within-subject factor is measurement time point (baseline, post-MPTP, 6-weeks post-MPTP, and 9-months post-MPTP).

Brain Tissue Harvest and Preparation

Brain tissue was harvested from four animals in each group at either six weeks or nine months after the last injection of MPTP for a total of 24 animals. Animals were sedated with ketamine (0.3ml of 100mg/ml) followed by a lethal dose of sodium pentobarbital (2 ml of 50 mg/ml solution) and monitored for eye reflex, breathing, and heartbeat. Upon cessation of vital signs, brains were quickly removed, briefly cooled on wet ice, and sectioned at 3-mm thickness in the coronal plane using an acrylic brain block designed specifically for the squirrel monkey brain starting at a position approximately 3 mm rostral to the midbrain. This resulted in 6 rostral coronal slices of 3 mm thickness. One fresh slice through the mid-striatum (at level A15 to A12 in the anterior-posterior plane) of each animal was used to free-hand dissect out the entire caudate nucleus or putamen, sectioned into 4 pieces in the dorsal-ventral plane, and snap frozen on dry ice to be used for either HPLC analysis of dopamine and its metabolites or protein analysis using western immunoblotting (Emmers and Akert 1963). The remaining caudal brain, which included the midbrain, was split at the mid-plane into two equal halves. Brain slices were immersion fixed in 4% paraformaldehyde in phosphate buffered saline (pH 7.4) for 48 hours, cryoprotected in 20% sucrose in phosphate buffer (pH 7.4) for 24 hours, and frozen onto microscope slides using immersion into isopentane on dry ice. For immunohistochemical staining, tissue was cut at 30-micron thickness

using a Leica 1950 cryostat. Sections were placed free-floating in phosphate buffered saline (pH 7.4) containing 0.01% thimerosal and refrigerated until used.

HPLC Analysis of Dopamine and its Metabolites

Neurotransmitter concentrations were determined according to an adaptation of Irwin et al (1992) of the method of Kilpatrick and colleagues (1986)(Irwin et al. 1992; Kilpatrick et al. 1986). Tissues for analysis were homogenized in 0.4 N perchloric acid and centrifuged at 12,000 x g to separate precipitated protein. The protein pellet was resuspended in 0.5N NaOH and the total protein concentration determined using the Coomassie Plus protein assay system (Pierce, Inc) using a Biotek Model Elx800 microplate reader and the software KCjunior. The concentrations of dopamine, DOPAC, HVA, were assayed by high performance liquid chromatography (HPLC) with electrochemical detection (ECD). Samples were injected with an ESA autosampler. Dopamine and its metabolites were separated by a 150 mm X 3.2 mm reverse phase 3 um diameter C-18 column (ESA, Chelmsford, MA) regulated at 28°C. The mobile phase MD-TM (from ESA) consisted of acetylnitrile in phosphate buffer and an ion-pairing agent delivered at a rate of 0.6 ml/minute. The electrochemical detector was an ESA model Coularray 5600A with a 4-channel analytical cell with set potentials at –100 mV, 50 mV, and 220 mV. The HPLC was integrated with a Dell GX-280 computer with analytical programs including ESA Coularray for Windows software and the statistics package InStat (San Diego, CA). Descriptive statistics were calculated for dopamine, DOPAC, HVA, and the turnover rate for each group-time point combination (control 6-weeks, control 9-months, 2-time MPTP 6 weeks, 2-time MPTP 9 months, 6-time MPTP 6 weeks, 6-time MPTP 9 months) and within four basal ganglia areas (dorsal putamen; dorsal caudate nucleus; ventral putamen; ventral caudate nucleus). Independent t-test revealed no differences between the saline groups for each of the four dependent measures and as such, the two saline groups were collapsed for further analysis. One-way ANOVA was use to evaluate differences between the group-time point combinations (control, 2-time MPTP 6 weeks, 2-time MPTP 9 months, and 6 times MPTP 9 months) in the levels of dopamine,

DOPAC, HVA, and turnover rate for each of the four basal ganglia regions (dorsal putamen; dorsal caudate nucleus; ventral putamen; ventral caudate nucleus).

Stereological Analysis of Nigrostriatal Neurons

The number of nigrostriatal dopaminergic neurons in the substantia nigra pars compacta (SNpc) was determined using unbiased stereology with the computer-imaging program BioQuant (BioQuant Image Analysis Inc, Nashville, TN) on a Dell computer and an Olympus BX-50 microscope equipped with a motorized stage and Retiga CCD camera. The hemi-sectioned brain (2 animals each from saline, 2-time and 6-time MPTP-injected) with its most rostral sections starting at level A9.0 was mounted in the cryostat at a temperature of -18^0C. Tissue was sliced at 30-micron thickness and every sixth section collected starting just prior to the rostral aspect of the substantia nigra (at level A8.0) completely through to the most caudal aspect when emergence of the pontine nucleus (at level A3.5) occurs. Tissue sections were stained for TH immunoreactivity and counterstained for Nissl substance. The SNpc was delineated from the rest of the brain based on TH-immunoreactivity and anatomical landmarks at both the rostral and caudal aspects. Each stained ventral mesencephalon section was viewed at low magnification (10X objective) and the SNpc outlined and delineated from the ventral tegmental-immunoreactive neurons using the third nerve and cerebral peduncle as landmarks. Neurons were viewed at high magnification (80X objective) and counted if they displayed TH-immunoreactivity and had a clearly defined nucleus, cytoplasm, and nucleolus. The total number of SNpc dopaminergic neurons was determined based on the method of Gunderson (Gundersen and Jensen 1987).

Immunohistochemical Analysis

The antibodies used in these studies were all purchased from Chemicon, Inc., (Temecula, CA) and included anti-tyrosine hydroxylase (polyclonal made in rabbit or monoclonal mouse), anti-dopamine transporter protein (monoclonal made in rat), and anti-DARPP-32 (polyclonal made in rabbit). Sections

were rinsed in TBS (50 mM Tris pH 7.4 with 0.9% NaCl), exposed to primary antibody (concentration of 1:1000) for 48 hours at 4°C, rinsed in TBS, and then exposed to secondary antibody made against the species of the primary antibody (using the ABC Elite Kit, Vectastain, Burlingame, CA). Antibody staining was visualized by development in diaminobenzoic acid/H_2O_2. To ensure that differences in staining intensity are in fact due to differences in antigen expression, multiple sections from each treatment group and time point were carried out concurrently under identical staining conditions. Specificity of antibody probes was verified by methods that eliminated staining including (i) omitting primary antibody, (ii) omitting secondary antibody, or (iii) omitting both primary and secondary antibodies. The relative intensity of labeling between stained sections in the different treatment groups was determined using computer assisted image analysis. Slides were simultaneously scanned on a high-resolution transmission light scanner (UMAX Powerlook 1120) and digitized images analyzed using NIH Image (version 1.66). Slide and section background (using the corpus callosum) were captured. Since sections for comparison were stained simultaneously within an experiment, variability in background was minimal. Comparison of immunostaining based on the relative optical density was determined by analyzing images using a region of interest encompassing the dorsal caudate nucleus or putamen. At least 6 to 8 sections from 2 or 3 animals were used for analysis.

Western Immunoblot Analysis

Western blotting was used to determine the relative protein expression in the basal ganglia. The immunoblotting technique was previously described (Jakowec et al. 1995). Tissue dissected from the caudate nucleus or putamen was homogenized in buffer (25 mM Tris pH 7.4, 1 mM EDTA, 100 uM PMSF, 0.1% SDS). Protein concentration was determined by the BCA method (Pierce, Inc). Equal amounts of protein (25 ug) were separated by the method of Laemmli (Laemmli 1970). Proteins were transferred to nitrocellulose filters by electroblotting in Towbin buffer (Towbin et al. 1979). Filters were blocked in TS-Blotto (50 mM Tris pH 7.4, 0.9% NaCl, 5% non-fat milk), then primary antibody (1:2000),

exposed to secondary antibody and visualized by chemiluminescence (Pierce, Inc). Filters were apposed to film (Hyperfilm ECL, Amersham, Inc) and processed in X-OMAT developer. Images were scanned into a computer using a Personal Laser Densitometer (Molecular Dynamics, Sunnyvale, CA) and the intensity of bands determined using computer assisted image analysis (NIH Image). The intensity of bands from western blot autoradiographs was expressed as relative optical density. Each treatment group's relative optical density was expressed as a percentage of the saline injected group. Group comparisons were made using one-way ANOVA with Bonferroni correction to determine statistical significance or effect size (meaningful difference) (Thomas et al. 1991). For all statistical tests, significance was set at $p < 0.05$. Post-hoc orthogonal contrasts with Bonferroni correction were preformed to determine the locus of any significant main and interaction effects.

RESULTS

Motor Behavioral Deficits After MPTP-Lesioning and the Time Course of Motor Behavioral Recovery

Motor behavior was assessed using a Clinical Rating Scale (CRS) as outlined in Table 1. The baseline CRS scores prior to either saline or MPTP administration for all groups of animals ranged from 1 to 2 out of a total score of 24. At baseline, all animals displayed normal cognition with a high degree of alertness, attentiveness, and motivated interactions with the rater. Throughout the entire study, saline-injected animals had a CRS score of 1.2 ± 0.2. Following completion of MPTP administration, all lesioned animals showed parkinsonian features including akinesia, bradykinesia, postural instability, and freezing. In addition, MPTP-lesioned animals were cognitively impaired and less attentive and required additional prompting for rater interactions. Two weeks after the last injection of MPTP, animals that received 2 injections of MPTP were mildly parkinsonian (8.3 ± 1.2), while animals that received 6 injections of MPTP were more severely parkinsonian (14.1 ± 1.2). The difference between the two

MPTP-lesioned groups was significant ($p < 0.0001$). The 6 times MPTP group was also cognitively more impaired than the 2-times MPTP group. At 6-weeks post-injection, both the 2-time and 6-time MPTP injection groups remained parkinsonian (CRS score of 4.9 ± 1.9 and 6.1 ± 15, respectively, $p < 0.0001$) (Figure 1). Both MPTP groups showed partial recovery compared to their respective CRS score at 2 weeks post-MPTP-lesioning with the 2-time injection group improving by 37.5% and the 6-time injection group improving by 57.0%, and this difference was significant ($p < 0.001$). At the 9-month time point, both the 2-time and 6-time MPTP-injection groups displayed complete motor behavioral recovery (CRS score of 1.8 ± 0.9 and 4.0 ± 2.3, respectively) and were not significantly different than the saline group. However, MPTP-lesioned animals continued to display cognitive impairments including poor rater interaction and motivation that was more evident in the 6-time MPTP-injected group.

Stereological Counting of SNpc Dopaminergic Neurons

Unbiased stereological counting methods were used to determine the degree of MPTP-lesioning based on number of TH-immunoreactive midbrain neurons. Saline animals had a total of 59,100 ± 4,440 TH-immunoreactive midbrain neurons while the 2-time MPTP-injected group had 64.5% depletion (to 21,000 ± 2,500) and the 6-time MPTP-injected group had an 81% depletion (to 11,300 ± 900).

HPLC Analysis of Dopamine and its Metabolites

Dopamine Levels at 6 Weeks after MPTP-Lesioning: HPLC analysis of dopamine and its metabolites (HVA and DOPAC) was performed on the dorsal and ventral regions of both the caudate nucleus and putamen. Levels of dopamine and turnover ratio, defined as {(DOPAC+HVA)/Dopamine} are shown in Table 2 and Figure 2. Since there was no significant difference between the saline groups at 6 weeks and 9 months, these data were combined for analysis. At 6 weeks post-MPTP injection, dopamine levels were significantly lower ($p < 0.001$) in the dorsal putamen of the 2-time injected (5.4 ± 5.2 ng dopamine / mg protein and 6-time injected (1.1 ± 0.3 ng dopamine / mg protein groups compared

to saline (161.1 ± 18 mg ng dopamine / mg protein). Dopamine levels at 6 weeks post-MPTP injection were also significantly lower ($p < 0.001$) in the dorsal caudate nucleus in the 2-time injected (6.6 ± 6.3 ng dopamine/mg protein and 6-time injected (0.6 ± 0.2 ng dopamine / mg protein groups compared to saline (95.0 ± 6.3 ng dopamine / mg protein). The degree of dopamine depletion in the dorsal caudate nucleus and putamen of the 6-time injected group was slightly greater than the 2-time injected group (see Table 2). Similar to the dorsal caudate nucleus and putamen, the ventral regions showed an analogous pattern of dopamine depletion. There was a significant depletion of dopamine ($p < 0.001$) in the ventral caudate nucleus in the 2-time injected group (16.6 ±16.1 ng dopamine / mg protein) and the 6-time injected group (5.9 ± 2.8 ng dopamine / mg protein) compared to saline (133.6 ± 25.0 ng dopamine / mg protein). The ventral putamen also showed significant dopamine depletion ($p < 0.001$) in the 2-time injected group (17.4 ± 16.9 ng dopamine / mg protein) and the 6-time injected group (12.1 ± 4.5 ng dopamine / mg protein) compared to saline (138.9 ± 25.4 ng dopamine / mg protein). Although it did not reach statistical significance, there was a trend for greater dopamine depletion in the dorsal regions of both the caudate nucleus and putamen compared to the respective ventral regions.

Dopamine Levels at 9 Months after MPTP-Lesioning: At 9 months after the last injection of MPTP, both the 2-time and 6-time MPTP-injection groups showed dopamine levels that were still severely depleted compared to the saline injected groups, showing only partial return. In the dorsal putamen, the level of dopamine remained significantly lower ($p < 0.001$) in both the 2-time (28.2 ± 11.1 ng dopamine / mg protein and 6-time injected (2.60 ± 0.3 ng dopamine / mg protein groups compared to saline (161.1 ± 18.1 ng dopamine / mg protein. In the dorsal caudate nucleus of the 2-time injected group (24.1 ± 6.4 ng dopamine/mg protein) showed reduced dopamine levels compared to the saline group (161.1 ±18.1 ng dopamine / mg protein but did not reach significance ($p < 0.06$). In addition, the level of

dopamine in the dorsal caudate nucleus remained significantly depleted ($p < 0.001$) in the 6-time injection (8.0 ± 4.0 ng dopamine / mg protein) groups compared to saline (95.0 ± 6.3 ng dopamine / mg protein).

The degree of dopamine return in the ventral regions of both the caudate nucleus and putamen was greater than that seen in the respective dorsal regions at 9 months after the last injection of MPTP. In the ventral putamen dopamine levels were significantly lower in the 6-time MPTP-injected group (35.8 ± 4.5 ng dopamine/mg protein) compared to saline (138.9 ± 25.4 ng dopamine / mg protein); however, in the 2-time MPTP injected group (83.9 ± 32.2 ng dopamine / mg protein) dopamine levels were not significantly different from the saline group (138.9 ± 25.4 ng dopamine / mg protein) showing a greater degree of return than the 6-time MPTP-injected group. In the ventral caudate, dopamine levels were significantly lower in the 6-time MPTP-injected group (31.5 ± 13.9 ng dopamine / mg protein) compared to saline (133.6 ± 25.0 ng dopamine / mg protein); however, dopamine levels in the 2-time MPTP injected group (83.9 ± 32.2 ng dopamine / mg protein) were not significantly different from the saline group (138.9 ± 25.4 ng dopamine / mg protein) again showing a grater degree of return compared to the 6-time MPTP-injected group.

Dopamine Turnover Ratio at 6 Weeks Post-MPTP Injection: In the dorsal putamen, the dopamine turnover rate was significantly increased ($p < 0.05$) 6 weeks after the last injection of MPTP in both the 2-time (75.3 ± 37.7) and 6-time MPTP-injected (44.9 ± 6.7) groups compared to saline (1.2 ± 0.2). In the dorsal caudate nucleus, the dopamine turnover ratio was significantly increased ($p < 0.05$) in both the 2-time (40.0 ± 21.5) and 6-time (52.3 ± 20.3) MPTP-injected group compared to saline (1.0 ± 0.1). In the ventral putamen, the turnover ratio remained elevated in the 2-time MPTP-injected group (98.6 ± 62.5) and to a lesser degree in the 6-time MPTP-injected group (11.5 ± 3.9) compared to saline (1.6 ± 0.2). In the ventral caudate nucleus, the turnover ratio was also elevated in the 2-time MPTP-

injected group (31.2 ± 18.6) and to a lesser degree in the 6-time MPTP-injected group (17.0 ± 11.9) compared to the saline group (1.1 ± 0.1).

Dopamine Turnover Ratio at 9 Months Post-MPTP Injection At 9 months after the last injection of MPTP, there was a decrease in the turnover ratio in both the 2-time and 6-time MPTP injected groups. In the dorsal putamen, the turnover ratio of the 2-time MPTP-injected group (5.6 ± 2.3) and 6-time (18.3 ± 9.2) MPTP injection animals showed a decrease, which was not significantly different from saline (1.2 ± 0.2). In the dorsal caudate nucleus, there was a decrease in turnover ratio in both the 2-time (3.7 ± 1.5) and 6-time (18.4 ± 13.4) MPTP injected groups, which was also not significantly different from saline (1.0 ± 0.1). In the ventral putamen, the turnover ratio also decreased in both the 2-time (2.8 ± 0.7) and 6-time (4.5 ± 2.0) MPTP-injected groups compared to saline (1.6 ± 0.2). In the ventral caudate nucleus, the turnover ratio decreased even more in both the 2-time (1.7 ± 0.4) and 6-time (4.9 ± 2.8) MPTP-injected groups compared to saline (1.1 ± 0.1).

Analysis of Tyrosine Hydroxylase (TH) Protein Expression

The pattern of expression of TH protein in the caudate nucleus and putamen of animals from all groups was determined using both immunocytochemistry and western immunoblotting (see Figure 4). In saline injected animals, TH-immunoreactivity (TH-ir) at the level of the mid-striatum showed intense staining throughout the entire caudate nucleus and putamen as seen in coronal sections at low magnification (Figure 4A). At high magnification the staining appeared as a dense fibrous network with staining in both puncta and dark fibers of various thicknesses (Figure 4B). At 6 weeks after the last MPTP injection, tissues from both the 2-time and 6-time MPTP-injected groups showed severe depletion of TH-ir. Remaining TH-ir appeared as light background staining with dark staining TH-ir fibers. The amount of TH-ir fibers was greater in the 2-time injection groups compared to the 6-time injected group (Compare Figure 4D and 4H). Analysis of TH protein at 9 months after the last injection of MPTP showed a partial

return of TH-ir in both the 2-time injection group (compare Figure 4C and E) and 6-time injection groups (compare Figure 4G and I). The degree of TH-ir return was greater in the 2-time injected group (compare Figure 4E and 4I) with the 2-time injected group showing a greater intensity of TH-ir (compare Figure 4F and 4J).

Western immunoblotting analysis was carried out to determine the relative degree of TH protein expression in the putamen from animals in all groups. A representative immunoblot is shown in Figure 5. There was no difference in the level of TH protein in saline animals harvested at 6 weeks or 9 months. Therefore, the results from the saline animals were pooled. The relative amount of TH protein at 6 weeks post-MPTP injection was significantly lower in the 2-time (16.2 ± 0.6% of saline) and 6-time (8.9 ± 1.1% of saline) MPTP injection groups compared to saline ($p < 0.01$). At the 9-month time point, the 2-time (31.8 ± 1.7% of saline) and the 6-time (20.7 ± 1.7% of saline) MPTP injected animal showed a slight increase in the relative amount of TH protein compared to their respective 6-week time point. This increase did not reach significance. However, the relative amount of TH protein at 9 months in both the 2-time and the 6-time MPTP injected animals remained significantly lower than saline animals ($p < 0.001$).

Analysis of Dopamine Transporter (DAT) Protein Expression

The pattern of expression of DAT protein in the caudate nucleus and putamen from animals in all groups was determined using both immunohistochemistry and western immunoblotting (see Figure 6). In saline injected animals, DAT-immunoreactivity (DAT-ir) in coronal sections at the level of the mid-striatum showed intense staining throughout as shown at low magnification (Figure 6A). At high magnification the staining appeared as a dense fibrous network similar to that seen with TH-ir with staining in both puncta and dark fibers of various thicknesses (Figure 6B). Tissues from both the 2-time and 6-time MPTP injection groups at 6 weeks post-injection of MPTP showed significant depletion of

DAT-ir. Remaining DAT-ir appeared as a light background staining with dark staining fibers. The degree of DAT-ir was greater in the 2-time injection group compared to the 6-time injected group (compare Figure 6D and 6H). At 9 months after the last injection of MPTP, analysis of DAT-ir showed a partial return in both the 2-time injected (compare Figure 6C and 6E) and 6-time injected groups (compare Figure 6G and 6I). The degree of DAT-ir return was greater in the 2-time injected group (compare Figure 6E and 6I) with the 2-time injected group showing a greater intensity of DAT-ir (compare Figure 6F and 6J).

Western immunoblot results supported findings for DAT using immunohistochemistry. At 6 weeks post-MPTP injection, both the 2-time and 6-time MPTP-injection groups were significantly reduced relative to the saline group (to 13.0 ± 1.3 % and 15.9 ± 3.2 % of saline, respectively, $p < 0.05$). At 9 months post-MPTP injection, there was a slight increase in DAT expression that was statistically significant in both the 2-time and 6-time MPTP injected groups (43.1 ± 2.8 % and 32.6 ± 5.0 % of saline, respectively, $p < 0.05$).

Analysis of DARPP-32 Protein Expression

Immunohistochemical analysis of the pattern of expression of DARPP-32 protein was carried out in coronal sections through the striatum of animals from all groups (See Figure 7). At 6 weeks after the last injection of MPTP both the 2-time and 6-time MPTP-injection groups showed reduced DARPP-32-immunoreactivity in cell bodies throughout the dorsal putamen to 76.4 ± 3.7 % and 63.2 ± 2.5% compared to the saline group, respectively ($p < 0.001$). At 9 months after the last injection of MPTP, DARPP-32-immunoreactivity in both the 2-time and 6-time injected groups returned to a level that was not significantly different from the saline group (101.1 ± 1.9 % and 91.9 ± 3.7 %, respectively).

DISCUSSION

The systemic administration of the neurotoxicant MPTP in the nonhuman primate squirrel monkey (*Saimiri sciureus*) leads to the selective destruction of dopaminergic neurons in the substantia nigra pars compacta and ventral tegmental area and the depletion of dopamine in the caudate nucleus and putamen (Burns et al. 1983; Langston et al. 1984). In addition to the neurochemical changes, MPTP-lesioned nonhuman primates display profound deficits in motor behavior that resemble features of parkinsonism including akinesia, bradykinesia (slowness), postural instability (balance), and freezing (Petzinger and Langston 1998). Similar to patients with idiopathic PD, MPTP-lesioned animals respond to dopamine replacement therapy (L-dopa plus carbidopa) with improvement of motor features and also display L-dopa related motor complications, including dyskinesia (Boyce et al. 1990a; Boyce et al. 1990b; Schneider 1989).

In our study, as in others, the degree of motor behavioral deficits is dependent upon the severity of MPTP lesioning since monkeys administered 2 injections of MPTP (2.0 mg/kg, free-base per injection) have mild symptoms while animals receiving a series of 6 injections of MPTP have more severe parkinsonism. Additionally, we observed that motor recovery was apparent by 6 weeks post-MPTP and was complete in both mild and severely lesioned animals by 9 months, with animals appearing indistinguishable from normal non-lesioned animals. Fully recovered animals display normal spontaneous movements (jumping and climbing throughout cage), as well as normal hand dexterity and balance. Behavioral recovery has been reported in several species of nonhuman primates following MPTP-lesioning (Elsworth et al. 1990; Elsworth et al. 2000; Kurlan et al. 1991; Rose et al. 1989a; Rose et al. 1989b). Interestingly, the reported degree of recovery is variable and may be dependent on several factors including the time after lesioning, the species, and the mode of behavioral assessment. For example, studies in the MPTP-lesioned marmoset showed recovery with respect to gross akinesia by 3 to 4 months post-lesioning but "lack of spontaneous movement and poor coordination of activities" were still evident and persisted at 18 months post MPTP (Rose et al. 1989a; Rose et al. 1989b; Ueki et al.

1989). In our study, motor deficits were not apparent by cage side examination by 9 months post-MPTP; however, cognitive deficits were noted even at the 9-month time point in both the 2-times and 6-times lesioned animals. Cognitive dysfunction without gross motor impairment has been reported in animals after MPTP lesioning, and may indicate either a greater vulnerability to injury of those dopaminergic pathways involved in cognitive processing and/or a diminished capacity to recover (Decamp and Schneider 2004; Schneider and Pope-Coleman 1995; Slovin et al. 1999; Taylor et al. 1990).

Using our MPTP lesioning regimen, we saw marked dopamine depletion in the caudate nucleus and putamen at 6 weeks that persisted at 9 months despite behavioral recovery. However, there were differences between dorsal and ventral regions within both the caudate nucleus and putamen. At 9 months after MPTP lesioning, this dorsal-ventral difference was most dramatic. For example, at 6 weeks after the last injection of MPTP while both groups of animals remained symptomatic, dopamine levels in the dorsal putamen were 93% (2 times group) and 99.3% (6 times group) depleted compared with 87% and 91% depletion, respectively, in the ventral putamen. At 9 months after the last injection of MPTP, when full motor recovery was observed, there was a partial return of dopamine levels that was more pronounced in the 2-time MPTP-injected group compared to the 6-time MPTP-injected group, and was more apparent in the ventral region (48.7% and 76% depletion compared to saline, respectively) compared to dorsal (72% and 98% depletion compared to saline, respectively). While there have been relatively few reports, our findings are similar to those that have demonstrated a persistent dopamine depletion in the presence of behavioral recovery as well as the greater return of dopamine in the ventral region (Gnanalingham et al. 1995; Rose et al. 1989a; Rothblat and Schneider 1995; Schneider and Rothblat 1991). Consequently, our work, like that of others, supports the hypothesis that other compensatory mechanisms may account for behavioral recovery with persistent dopamine depletion (Eidelberg et al. 1986; Gnanalingham et al. 1995; Schneider 1992). Where we differ from previous work is in our findings of compensatory mechanisms that have been suggested, namely (i) turnover rate, (ii) the

degree of expression of the dopamine transporter protein during recovery, and (iii) sprouting of surviving dopaminergic neurons as indexed by the return of expression of TH protein.

Turnover ratio is an indicator of one potential compensatory mechanism accounting for behavioral recovery despite persistent striatal dopamine depletion (Rose et al. 1989b). Dopamine turnover is the ratio of dopamine metabolites to dopamine {(DOPAC + HVA) / dopamine}. The mechanism by which turnover could compensate is via the increased use of synaptic dopamine when there are low overall amounts of dopamine. Therefore, what has been suggested is an inverse and almost linear relationship between absolute striatal dopamine levels and turnover ratio: less dopamine leads to greater turnover ratio (Elsworth et al. 2000). The most striking finding in our study was that turnover ratio was more related to the time course of behavioral recovery than to the degree of lesioning or the amount of dopamine return. At the earliest time point (6 weeks after the last injection of MPTP) dopamine turnover rate was elevated in both the 2-time and 6-time MPTP-injected groups. However, at 9 months after the last injection of MPTP when dopamine levels remained depleted, turnover normalized in the 2-time MPTP-injected group and was markedly down in the 6-time MPTP-injected group. Therefore, our study indicates that increased turnover ratio may reflect an early mechanism of compensation after injury. In addition, the elevation in turnover ratio does not appear dependent on severity since the early increase in turnover ratio was often more pronounced in the 2-time compared with the 6-time group. Finally, the turnover ratio was not unequivocally associated with the amount of dopamine. For example, in the dorsal regions of both groups a decrease in turnover ratio with time was not accompanied by a pronounced increase in the amount of dopamine. Even in the ventral regions where turnover ratio is dramatically decreasing over-time we do not see a complete return of dopamine. Therefore, the compensation offered by turnover may not fully account for complete motor behavioral recovery, because turnover ratio is decreasing with behavioral recovery even though dopamine depletion persists.

Similar to our work, Jenner and colleagues found that return of dopamine was greatest in the ventral aspect of the striatum (Gnanalingham et al. 1995; Rose et al. 1989b). This latter finding may be

due to the fact that the ventral aspect of the striatum is near the medial forebrain bundle and may represent a site of robust midbrain sprouting from the nigrostriatal and/or ventral tegmental area neurons. Unlike our work, Jenner and colleagues observed a complete return of dopamine specifically in the nucleus accumbens with persistence of 90% depletion within the rest of the striatum. Additionally, unlike our findings, the persistent depletion of dopamine in the rest of the striatum was accompanied by an increased time in turnover ratio (1-year post-MPTP) in the caudate nucleus and putamen of the MPTP-lesioned marmoset. They suggest that behavioral recovery in the MPTP-lesioned marmoset was largely the result of these two phenomena, i.e. the increase in dopamine in the nucleus accumbens and increased turnover ratio within the striatum. However, since these animals continued to show lack of spontaneous movements and poor coordination we would argue that these mechanisms do not sufficiently explain motor recovery (Ueki et al. 1989).

The dopamine transporter (DAT) is a protein localized on nigrostriatal terminals and plays a role in dopamine uptake and diffusion therefore influencing dopamine synaptic occupancy (Gainetdinov et al. 2002). A potential mechanism that has been suggested as explaining behavioral recovery in the context of persistent dopamine depletion, most evident in the dorsal region, is the decreased expression of the dopamine transporter protein within the ventral striatum leading to the diffusion of dopamine to depleted regions (Rothblat and Schneider 1999; Schneider et al. 1994). As opposed to a decreased expression of DAT protein, we found an increased expression over time (9 months) during recovery. Therefore our data do not support a preferential decrease in uptake (and diffusion) as a mechanism of behavioral recovery.

Tyrosine hydroxylase (TH) is a enzyme important in the biosynthesis of dopamine and is often considered a marker of nigrostriatal neuron sprouting (Bezard et al. 2000; Jakowec et al. 2004; Rothblat et al. 2001). At 6 weeks post-MPTP lesioning, when dopamine levels are low, TH-immunoreactivity is severely depleted. At 9 months when animals show normal motor behavioral recovery, both dopamine levels and TH-immunoreactivity show only partial return. Our findings and those of others suggest that

sprouting of surviving nigrostriatal dopaminergic neurons may not be sufficient to account for motor recovery in the MPTP-lesioned squirrel monkey (Rothblat and Schneider 1994; Schneider et al. 1998). Other morphological approaches are needed to fully address this possibility.

In summary, our findings suggest that compensatory mechanisms associated with the dopaminergic system do not fully account for the complete motor recovery in the MPTP-lesioned squirrel monkey. This raises the possibility that other non-dopaminergic systems may be involved including those using glutamate, serotonin, gamma-amino-benzoic acid (GABA), and small neuropeptides such as enkephalin. While our studies were not directed at supporting the existence of compensatory mechanisms outside the dopaminergic system, our results with DARPP-32 suggest that the glutamatergic system may in fact play a role in motor recovery. We found that DARPP-32-specific staining was initially reduced (6 weeks post-MPTP) in striatal cells in both groups of animals, regardless of degree of lesioning. However, by the late time point DARPP-32 staining had fully returned throughout the striatum despite a persistent depletion of dopamine. DARRP-32 is an important downstream effector molecule within medium spiny neurons that is mediated through both glutamatergic (corticostriatal and thalamostriatal) and dopaminergic (nigrostriatal) afferents (Greengard et al. 1999; Nishi et al. 2005). Therefore, the return of DARRP-32 supports a greater role of the glutamatergic system in motor recovery of the MPTP-lesioned squirrel monkey in the face of persistent loss of dopaminergic input. Studies in rodent models of dopamine depletion and in primates support the role of glutamate in recovery process (Bezard et al. 1997; Bezard et al. 1999; Robinson et al. 2003). In our lab, this hypothesis is being tested through the administration of specific glutamate receptor antagonists to block glutamate neurotransmission and attenuate the return of DARPP-32 expression as well as to determine altered expression of glutamate receptor subunits.

In conclusion, findings from this study indicate that after MPTP-lesioning, dopamine return and compensatory mechanisms associated with the dopaminergic system are not sufficient to explain motor

recovery in the squirrel monkey. We suggest that non-dopaminergic systems, such as the glutamatergic system, may play an important role in recovery. In addition, our study demonstrates that the recovery process is dynamic given the changes in mechanism(s), such as turnover ratio, over time. These findings suggest two important treatment considerations of basal ganglia disorders such as Parkinson's Disease; (i) the existence of non-dopaminergic therapeutic targets, and (ii) the possibility that these targets may change in importance given the dynamic nature of the recovery process.

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TABLE and FIGURE LEGENDS

Table 1: ***The Modified Monkey Clinical Rating Scale.*** The rating scale is based on motor features of the Unified Parkinson's Disease Rating Scale (UPDRS) and employs items related to motor behavior in the squirrel monkey. The maximum total score is 24 points, which is derived from scores of 0 to 4 for six motor items. The "*" symbol indicates statistical significance compared to the saline control group ($p < 0.05$) and the "#" symbol indicates statistical significance between the 6-week and 9-months time points within a group ($P < 0.05$).

Table 2: ***HPLC Analysis of Dopamine and its Metabolites.*** The average amount of dopamine and its metabolites DOPAC and HVA were determined in tissues derived form the dorsal and ventral regions of both the caudate nucleus and putamen of at least 3 animals in each group. Tissue was collected at either 6 weeks or 9 months after the last MPTP injection from animals groups receiving 2 or 6 injections of MPTP. Data from saline–injected animals were pooled since statistical analysis of dopamine and its metabolites showed no differences in all regions and at 6 weeks and 9 months (resulting in $n = 6$). The turnover ratio is defined as (DOPAC + HVA)/DA. The "*" symbol indicates statistical significance from the saline group at $p < 0.05$.

Figure 1: ***Time Course of Motor Behavior.*** The Clinical Rating Scale score for saline- and MPTP-injected groups is shown at baseline (2 weeks prior to MPTP-injection), and at post-lesioning time points 1-week, 6-weeks, and 9-months after the last injection of MPTP. The "*" symbol indicates statistical significance compared to the saline CRS score at $p < 0.05$. The "#"symbol indicates statistical significance compared to the 2x-MPTP-injected group CRS score at $p < 0.05$.

Figure 2: ***Neurochemical Analysis of Dopamine and its Metabolites.*** Data are shown for the analysis of dopamine from (A) the dorsal putamen and (C) the dorsal caudate nucleus from N = 3 animals per group except saline where N = 6. Saline groups consist of pooled data from tissues collected at both the 6-week and 9-month time points. Tissue for analysis was collected from both the 2-time and 6-time MPTP-injected groups at either 6 weeks or 9 months after the last injection of MPTP. Dopamine turnover defined as (DOPAC and HVA)/DA, is depicted in (B) for the dorsal putamen and (D) the dorsal caudate nucleus. The "*"symbol indicates statistical significance compared to saline ($p < 0.05$). The symbol "#" indicates statistical significance compared to the 2-time MPTP injected group at 6 weeks ($p < 0.05$).

Figure 3: ***Immunohistochemical staining for tyrosine hydroxylase protein in the Substantia Nigra pars compacta.*** Representative sections are shown through the mid-SNpc immuno-stained for TH and Nissl substance comparing saline-injected and 6-time MPTP injected groups 9 months after the last injection. The upper panels are photomicrographs at low magnification while the lower panes are high magnification. Note the severe depletion of TH-immunoreactive cells in the SNpc in the MPTP-lesioned SNpc despite the complete return of motor behavior. The scale bar in the upper panels represents 800 microns while the scale bar in the lower panel represents 40 microns. SNpc = substantia nigra pars compacta.

Figure 4: ***Immunohistochemical staining for tyrosine hydroxylase protein in the caudate nucleus and putamen.*** The top panel shows representative images at low magnification of hemi-sections in the coronal plane at the level of the mid-striatum at the anterior-posterior plane between 14 and 15 mm. The lower panels represent high magnification images obtained from the most dorsal quadrant of the caudate

nucleus. (A) TH-ir is seen as dark immuno-staining throughout the caudate nucleus and putamen. (B) At higher magnification dark TH-ir appears as a thick fibrous network. (C) At 6-weeks following 2 injections of MPTP there is a significant reduction in TH-ir throughout the caudate nucleus and putamen and (D) only a light fibrous TH-ir network remains. (E) At 9 months after the last of the 2-time MPTP injections TH-ir increases throughout the striatum and (F) an increase in the fibrous network is observed. (G) At 6 weeks after the last injection in the 6-time MPTP regimen results in a severe depletion of TH-ir (H), which appears as very light staining with a small number of distinct fibers remaining. (I) At 9 months after the last of the series of 6 injections of MPTP there is an increase in the degree of TH-ir (J) corresponding to an increase in the degree of TH-ir fiber density. CN = caudate nucleus; Pu = putamen; cc = corpus callosum; ctx = cortex. The scale bar in G represents 0.5 mm for images in the top row and the bar in H represents 15 microns for images in the bottom row.

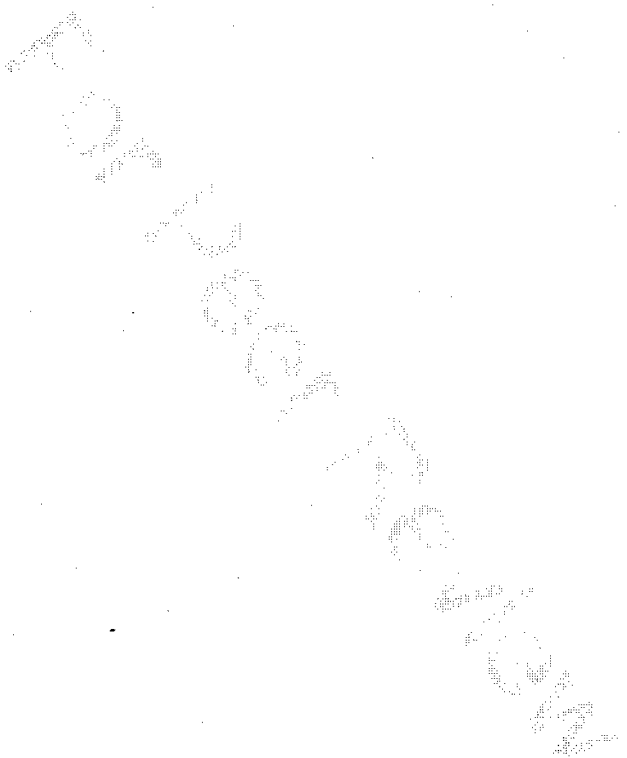
Figure 5: Western Immunoblot for Tyrosine Hydroxylase Protein in the Caudate Nucleus. The upper panel shows a representative immunoblot comparing the degree of TH protein expression in the caudate nucleus from tissues collected from the 2-time and 6-time MPTP-injected groups at 6 weeks and 9 months after the last injection. Note the highest amount of expression in the saline group, depletion at 6 weeks in both MPTP-lesioned groups and partial return in both MPTP-lesion groups at 9 months. The lower panel shows the relative optical density (OD) of immunoblot data derived from 2 samples from 3 different animals from each group. All MPTP-lesioned groups indicated by the "*" symbol were statistically significant from saline ($p < 0.05$).

Figure 6: ***Immunohistochemical Staining for Dopamine Transporter in the Caudate Nucleus and Putamen.*** Coronal sections through the mid-striatum at level in the anterior-posterior plane at 14 to 16

mm were stained for DAT-ir and representative sections photographed at low magnification (12.5X in the upper panels) and at high magnification (400X in the lower panels). (A) Sections from saline injected animals showed intense dark staining throughout the caudate nucleus and putamen and (B) appeared as a dark fibrous network with a high background. (C) At 6 weeks after the last injection in the 2-time MPTP regimen DAT-ir was significantly reduced with (D) a small number of dark DAT-ir fibers remaining on a lightly immunoreactive background. (E) After 9 months the 2-time MPTP injection regimen showed the return of DAT-ir that (F) appeared as a fibrous network of various sizes with increased DAT-ir background. (G) At 6 weeks following the regimen of 6 injections of MPTP DAT-ir was significantly reduced throughout the caudate nucleus and putamen with (H) a small number of DAT-ir fibers remaining. (I) At 9 months following the regimen of 6 injections of MPTP there was a slight return of DAT-ir that (J) appeared as an increase in the staining of fibers of various sizes resulting in increased background staining. The "*" symbol indicates statistical significance compared to the saline control group ($p < 0.05$) and the "#" symbol indicates statistical significance compared to the 6-week time point within each group ($P < 0.05$).

Figure 7: ***Immunohistochemical Staining for DARPP-32 Protein in the Caudate Nucleus and Putamen.*** Coronal sections through the mid-striatum were stained for DARPP-32 immunoreactivity and representative sections photographed at low magnification (12.5X in the upper panels) and at high magnification (400X in the lower panels). (A) Saline animals showed immunoreactivity throughout the striatum at low magnification, (B) which appeared as staining predominantly within cell nuclei and to a lesser extent in the neuropil. At 6 weeks after MPTP-lesioning both the 2-time and 6-time injected animals showed a reduction in the intensity of overall immunoreactivity (C and G), which appeared as reduced staining within both cell bodies themselves and to a lesser degree the neuropil (D and H). Immuno-staining was less intense in the 6-time injected group compared to the 2-time injected group (D

and H). At 9 months after MPTP-lesioning immuno-staining in the 2-time injection group was similar to that seen in saline animals (E and F). Immuno-staining at 9 months after MPTP-lesioning in the 6-time injection group had immuno-staining (I and J) that was less intense then both the saline and 2-time injected animals at the same time point. The graph depicts the relative optical density of DARPP-32 immuno-staining in the dorsal caudate nucleus in all treatment groups (n = 8 sections per group). The "*" symbol indicates statistical significance compared to the saline control group ($p < 0.05$).



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Parkinsonian Clinical Rating Scale Modified for Squirrel Monkey

Parkinsonian Motor Features:

1. **SPATIAL HYPOKINESIA** (Movement around cage)
   - 0- Normal (uses entire cage space)
   - 1- Utilizes most of the cage (at least 75% of cage space), but may be slow.
   - 2- Definitely slowed, but uses more than 50% of cage space.
   - 3- Definitely slowed, using less than 50% of cage space.
   - 4- Does not move from a confined area, with little or no movement

2. **BODY BRADYKINESIA**
   - 0- Normal movement around cage or bars
   - 1- Slow or deliberate body movements, could be normal for age.
   - 2- Moderately slow, intermittent limb dragging, moves without provocation.
   - 3- Marked slowness, requires provocation to move arms or legs.
   - 4- Frozen, little or no body movements regardless of provocation.

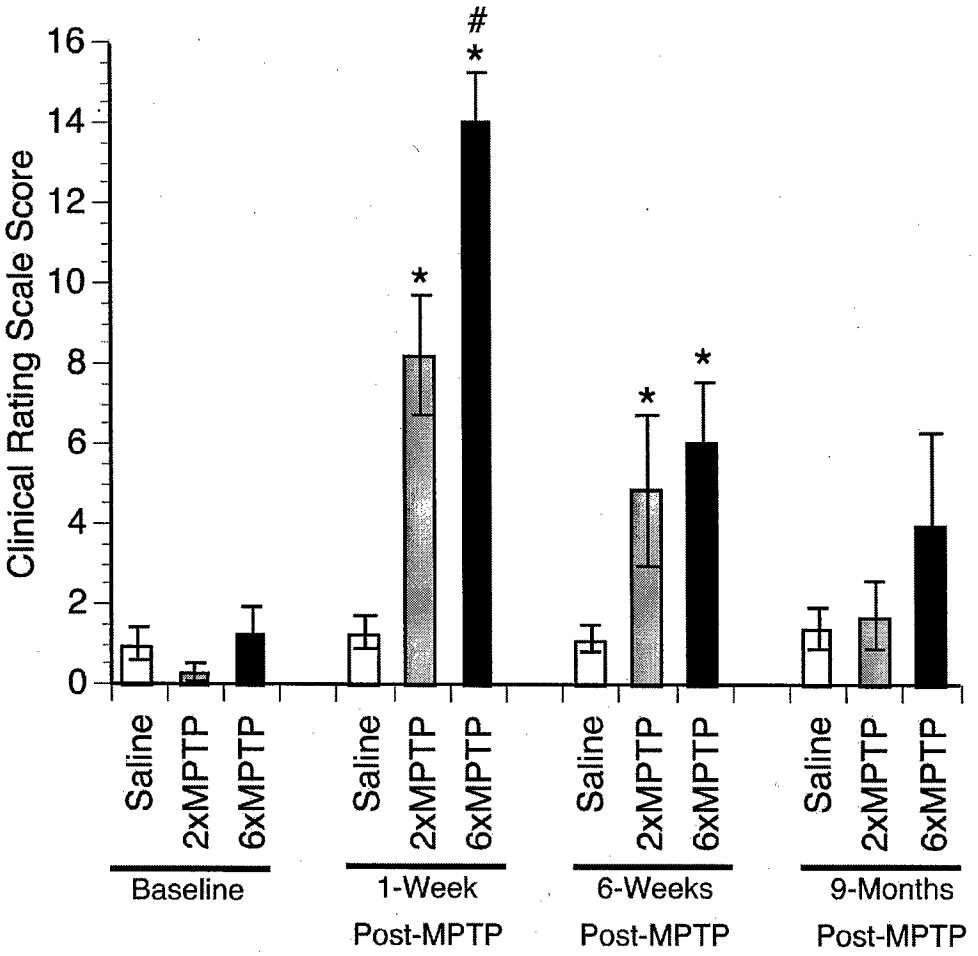
3. **MANUAL DEXTERITY (right arm/)**
   - 0- Normal
   - 1- Mildly slow or some loss of maneuverability of food items, could be normal for age.
   - 2- Moderate slowness, noticeable effort needed to grab or maneuver food.
   - 3- Marked slowness, with multiple attempts needed to grab food, may use both hands, may drop food.
   - 4- Severe slowness, with inability to grab or maneuver food, may need to be hand fed.

4. **MANUAL DEXTERITY (left arm/)**
   - 0- Normal
   - 1- Mildly slow or some loss of maneuverability of food items, could be normal for age.
   - 2- Moderate slowness, noticeable effort needed to grab or maneuver food.
   - 3- Marked slowness, with multiple attempts needed to grab food, may use both hands, may drop food.
   - 4- Severe slowness, with inability to grab or maneuver food, may need to be hand fed.

5. **BALANCE**
   - 0- Normal
   - 1- Slight tendency to hold on to cage, may be normal for age or no falls
   - 2- Uses both hands intermittently for support or rare occasional falls
   - 3- Uses both hands for support at all times or frequent falls
   - 4- Continually hanging on for support or falls with any attempt to move

6. **FREEZING: Observation over 4 minute Clinical Evaluation**
   - 0- None, no freezing ever observed
   - 1- Occasional mild (< 5 sec) freezing episodes
   - 2- Mild freezing episodes < 5 sec duration, or rare severe (> 5 sec)
   - 3- Frequent severe freezing observed > 5 sec
   - 4- Frozen most of the time

Treatment	Time Point	Region		Dopamine (ng/mg protein)	DOPAC (ng/mg protein)	HVA (ng/mg protein)	Turnover Rate
Saline		Caudate	Dorsal (N = 6)	95.0 ± 6.3	11.7 ± 1.7	81.6 ± 10.5	1.0 ± 0.1
			Ventral (N = 6)	133.6 ± 25.0	15.0 ± 1.2	121.7 ± 20.4	1.1 ± 0.1
		Putamen	Dorsal (N = 6)	161.1 ± 18.1	23.5 ± 4.0	168.2 ± 15.6	1.2 ± 0.2
			Ventral (N = 6)	138.9 ± 25.4	22.0 ± 5.1	176.2 ± 13.9	1.6 ± 0.2
2 X MPTP	6 Weeks	Caudate	Dorsal (N = 3)	6.6 ± 6.3*	1.7 ± 1.5	29.3 ± 17.6	40.0 ± 21.5
			Ventral (N = 3)	16.6 ± 16.1*	2.8 ± 2.2	29.3 ± 11.6	31.2 ± 18.6
		Putamen	Dorsal (N = 3)	5.4 ± 5.2*	1.2 ± 1.1	41.5 ± 19.8	75.3 ± 37.7*
			Ventral (N = 3)	17.4 ±16.9*	3.4 ± 2.9	63.2 ± 14	98.6 ± 62.5*
	9 Months	Caudate	Dorsal (N = 3)	24.1 ± 6.4	9.8 ± 3.5	61.6 ± 8.2	3.7 ± 1.5
			Ventral (N = 3)	68.6 ± 18.7	15.0 ± 5.7	88.9 ± 7.5	1.7 ± 0.4
		Putamen	Dorsal (N = 3)	28.2 ± 11.1*	8.5 ± 3.8	102.8 ± 24.7	5.6 ± 2.3#
			Ventral (N = 3)	83.9 ± 32.2	14.3 ± 4.0	174.4 ± 31.2	2.8 ± 0.7
6 X MPTP	6 Weeks	Caudate	Dorsal (N = 3)	0.6 ± 0.2*	0.5 ± 0.1	22.7 ± 3.5	52.3 ± 20.3
			Ventral (N = 3)	5.9 ± 2.8*	2.4 ± 0.4	36.9 ± 2.6	17.0 ± 11.9
		Putamen	Dorsal (N = 3)	1.1 ± .3*	0.9 ± 0.3	42.5 ± 11.8	44.9 ± 6.7
			Ventral (N = 3)	12.1 ± 4.5*	6.8 ± 2.9	98.9 ± 17.2	11.5 ± 3.9
	9 Months	Caudate	Dorsal (N = 3)	8.0 ± 4.0*	2.6 ± 0.7	73.4 ± 29.5	20.9 ± 12
			Ventral (N = 3)	31.5 ± 13.9*	10.2 ± 2.3	89.2 ± 26.0	5.4 ± 2.5
		Putamen	Dorsal (N = 3)	2.6 ± .27*	2.12 ± .07	55.7 ± 10.3	23.2 ± 5.7
			Ventral (N = 3)	35.8 ± 14.2*	11.8 ± 0.82	71.3 ± 25.6	4.8 ± 1.8



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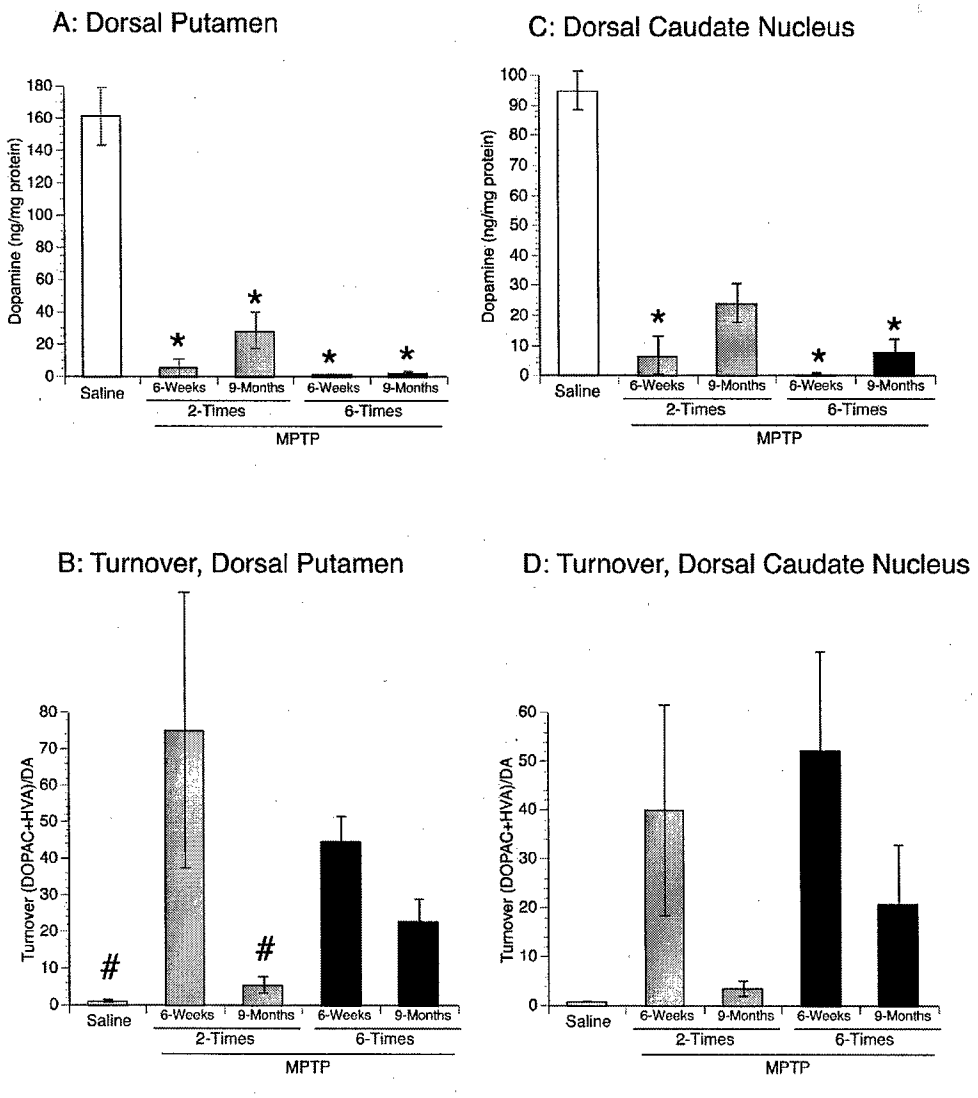

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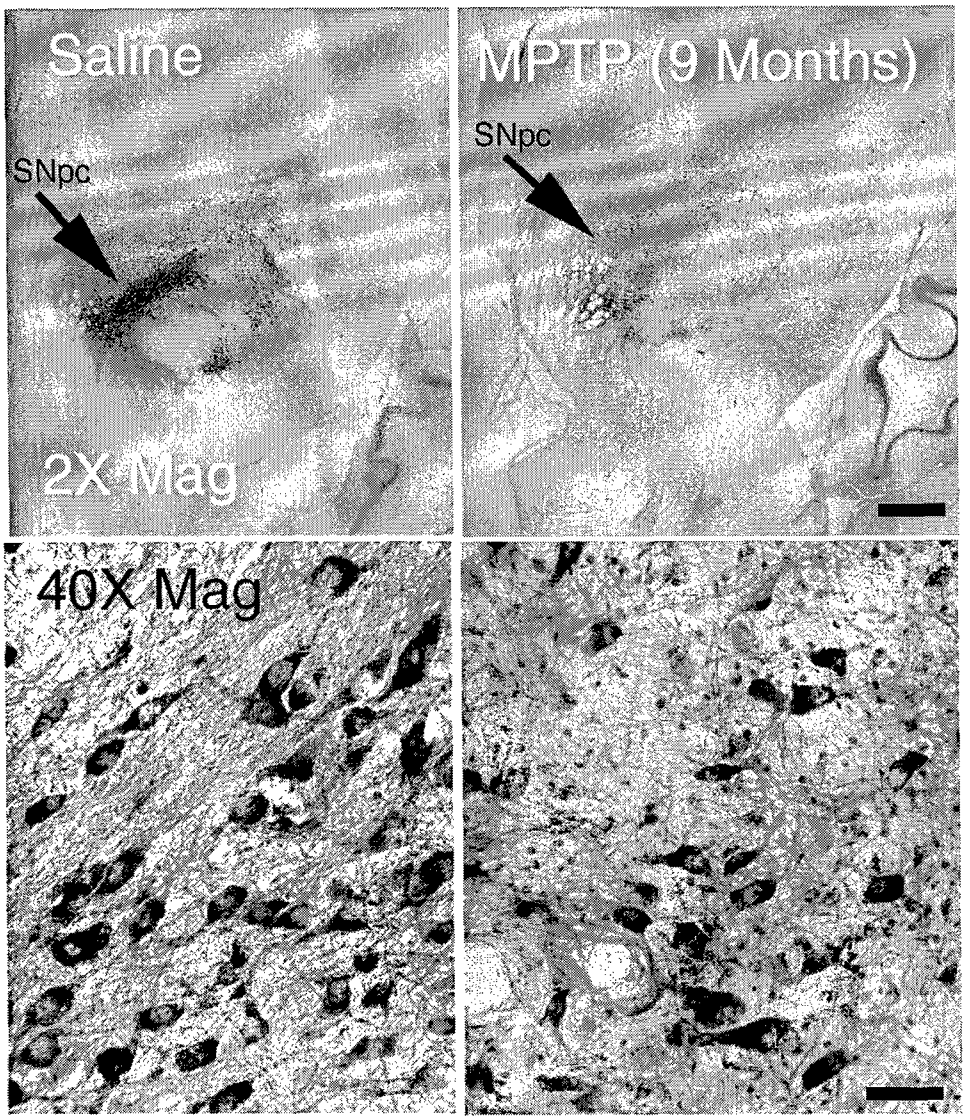

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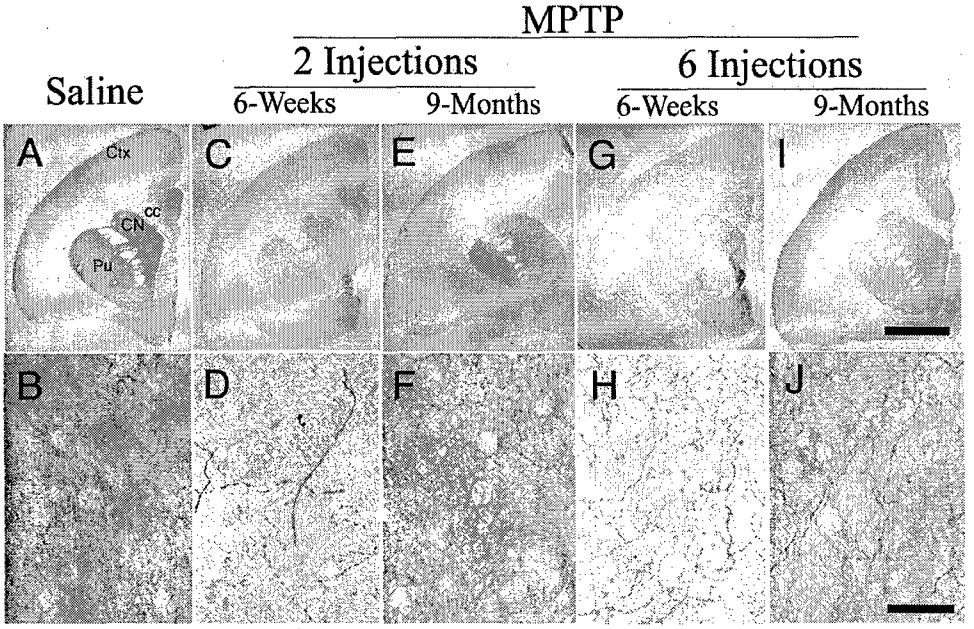

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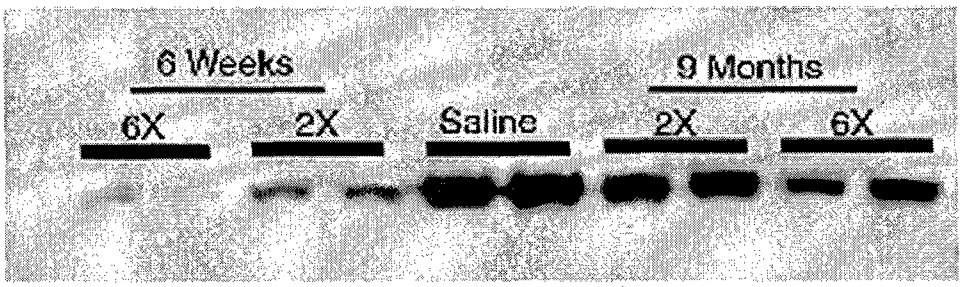

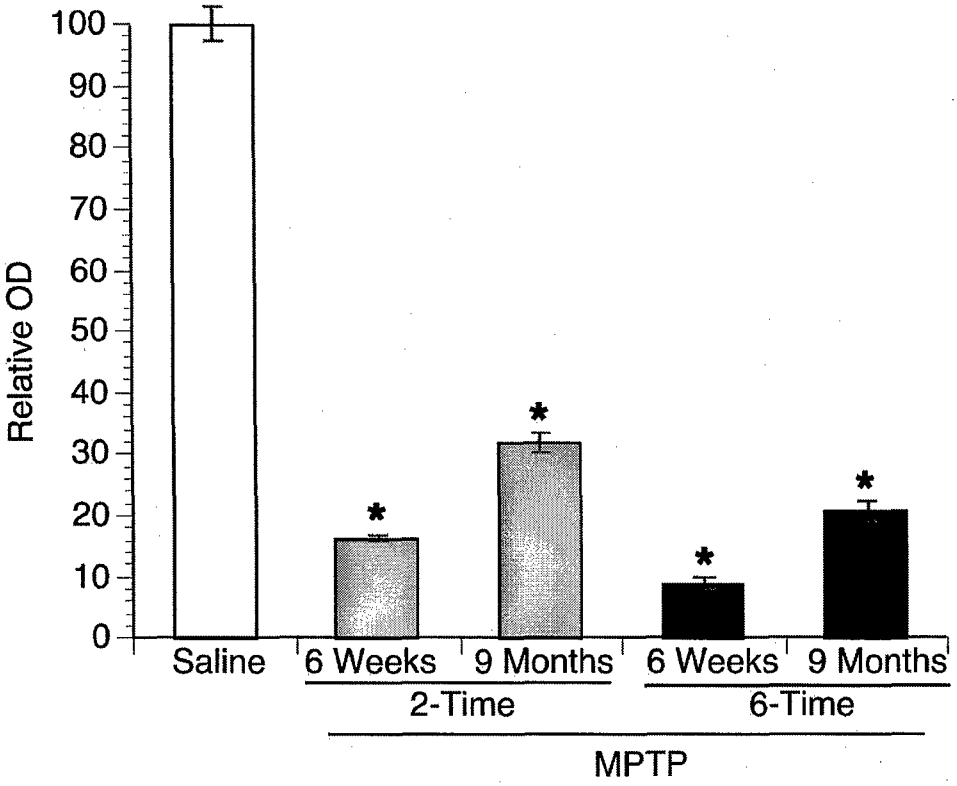

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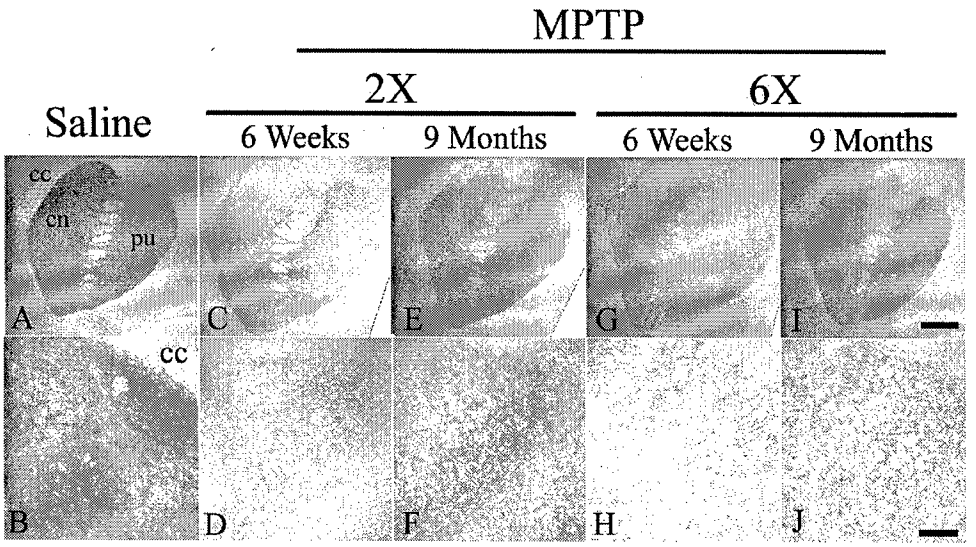

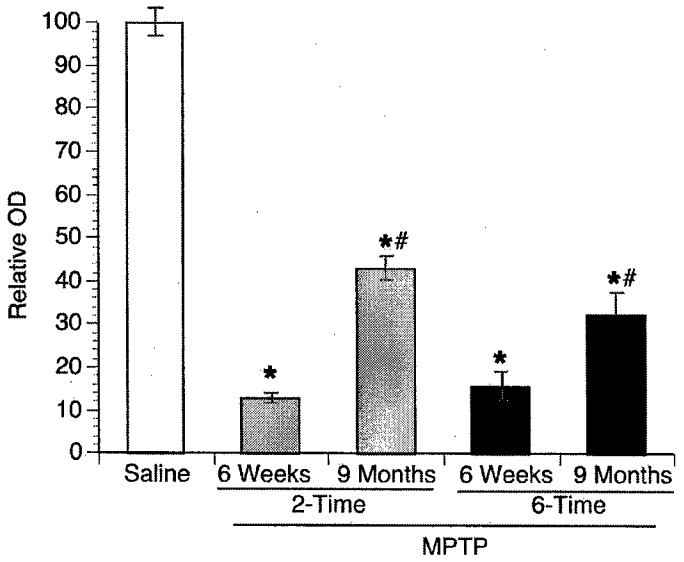

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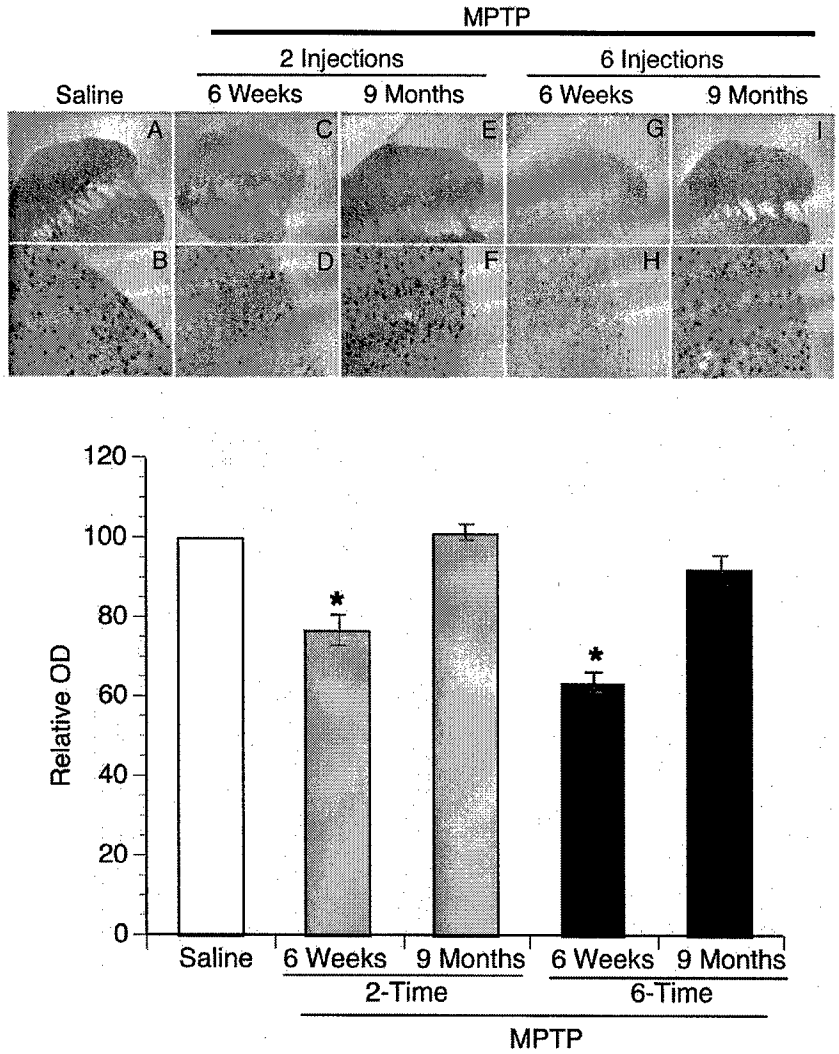

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Neuroplasticity and Treadmill Exercise in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-(MPTP)-Lesioned Mouse Model: Analysis of Striatal Dopamine, and Midbrain mRNA transcripts for Tyrosine Hydroxylase and Dopamine Transporter.

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Number of Tables: 1
Number of Figures: 3

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ABSTRACT (max 250 words)

Using a treadmill exercise paradigm, we investigated the effect of exercise on neurorestoration in the MPTP-lesioned mouse model of basal ganglia injury. C57BL/6J mice were administered MPTP (4 injections of 20 mg/kg free-base, 2 hours apart) or saline and divided into the following groups: (i) saline; (ii) saline + exercise; (iii) MPTP; (iv) MPTP + exercise. Mice in exercise groups were run on a motorized treadmill for 28 days starting 4 days after MPTP-lesioning; a period after MPTP-induced cell death is complete. Both running speed and endurance improved in the MPTP + exercise group to near normal levels over the 28-day exercise period. Exercise resulted in a significant down-regulation of striatal tyrosine hydroxylase and dopamine transporter in the MPTP + exercise group compared to MPTP non-exercised group both after 7 and 28 days of exercise. Analysis of striatal dopamine showed that MPTP + exercise mice did not have significantly different levels of dopamine compared to MPTP non-exercise group despite enhanced motor behavioral recovery in the former group. Additional analysis of mRNA transcript expression in surviving nigrostriatal dopaminergic neurons showed that there was no significant difference in tyrosine hydroxylase and dopamine transporter between MPTP exercise and non-exercise mice. These results indicate that enhanced recovery in MPTP + exercise mice occurs despite reduced expression of striatal dopamine and proteins involved in dopamine neurotransmission. Therefore, in agreement with our previous studies, non-dopaminergic systems may play a compensatory role in exercise-induced neuroplasticity in the injured basal ganglia.

Keywords: in situ hybridization, nigrostriatal, basal ganglia, Parkinson's disease.

Theme: Disorders of the Nervous System

Topic: Neurodegenerative and Movement Disorders: Parkinson's Disease Models

Body of Text (max 5000 words or 5000 minus 250 words per Figure)

The adult nervous system is capable of tremendous plasticity in both the non-injured and injured state. Similar to mechanisms involved in early postnatal activity-dependent neurodevelopment, the adult central nervous system can be influenced by environmental interactions. One such interaction is through environmental enrichment including exercise. There is increased interest in the role of environmental enrichment plays in facilitating neuroplasticity that can be both neuroprotective and neurorestorative in neurodegenerative diseases. In rodent models, environmental enrichment has been shown to be neuroprotective when introduced either before or during neurotoxic insult to the basal ganglia (REF; Tillerson; Bezard). In addition, studies in our lab and that of others have also shown that exercise can influence neurorestoration of motor behavior following the completion of nigrostriatal dopaminergic neuron death using 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (REF; Fisher; Tillerson).

The interest in our laboratory is to investigate the role of intensive treadmill exercise in promoting repair of the injured basal ganglia after MPTP-lesioning. Previous studies have shown that treadmill exercise enhances motor behavioral recovery in the C57BL/6J MPTP-lesioned mouse (REF; Fisher). Initially we speculated that exercise-enhanced behavioral recovery involve an acceleration of the intrinsic neuroplasticity which underlies the return of striatal dopamine and tyrosine hydroxylase (TH) and dopamine transporter (DAT), two markers of the integrity of the nigrostriatal dopaminergic system (REF; Jakowec 2004; Bezard; Chuih). We found that exercise leads to the suppression of both striatal TH and DAT protein expression despite enhanced recovery. Interestingly, we also found that exercise alters the MPTP-dependent alterations in synaptic glutamate. The purpose of this study was to determine if intensive treadmill exercise in the MPTP-lesioned mouse model of basal ganglia injury leads to increased production of striatal dopamine by surviving nigrostriatal dopaminergic neurons.

Furthermore we wished to know if these neurons within the substantia nigra display altered expression of TH and DAT mRNA transcripts in response to exercise.

MATERIALS AND METHODS

Mice used for these studies were young adult (8 to 10 weeks old) male C57BL/6J mice supplied from Jackson Laboratory, Inc. (Bar Harbor, Maine). There were 4 treatment groups including: (i) saline-injected, (ii) saline + exercise, (iii) MPTP-lesioned, and (iv) MPTP-lesioned + exercise. Animals were housed 6 to a cage and acclimated to a 12-hour shift in light/dark cycle so that the exercise occurred during the animals normal wake period. All experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised 1996) and approved by the University of Southern California Institutional Animal Care and Use Committee (IACUC).

MPTP (Sigma, Inc, St. Louis, MO) was administered in a series of 4 intraperitoneal injections of 20 mg/kg (free-base) at 2-hour intervals for a total administration of 80 mg/kg. This regimen leads to a 60 to 70% loss of nigrostriatal neurons (as determined by unbiased stereological techniques for both TH staining and Nissl substance and an 80-90% depletion of striatal dopamine levels [Jakowec, 2004 #4800][Jackson-Lewis V, 1995 #696]. Nigrostriatal cell loss is complete by day 3 after MPTP administration and there is no change in nigrostriatal tyrosine hydroxylase immunoreactive cell numbers during the exercise study period [Jakowec, 2004 #4800].

Sixty animals that could maintain a forward position on the 45-cm treadmill belt for 5 minutes at 5.0 m/min were randomly assigned to the 4 groups. The treadmill used in these studies was a Model

EXER-6M Treadmill manufactured by Columbus Instruments (Columbus, Ohio). Prior to MPTP lesioning, a baseline treadmill running assessment was conducted to insure that all animals performed similarly on the treadmill task prior to MPTP lesioning. A non-noxious stimulus (metal beaded curtain) was used as a tactile incentive to prevent animals from drifting back on the treadmill. As a result, shock-plate incentive was not used and stress related to the activity was minimized. Exercise was initiated 4 days following saline or MPTP-lesioning when cell death is complete. Mice from each of the two exercise groups (saline + exercise and MPTP + exercise) were run at the same time in the 6-lane treadmill. Exercise duration was incrementally increased starting with 30 minutes on day 1 to reach the goal duration of 2 sessions of 30 minutes each (for a total of 60 minutes), 5 days/week (with a 5 minute warm-up period) for a total of 28 days of exercise. Treadmill speed and exercise duration for each group was increased when all 6 mice within each group maintained a forward position on the treadmill belt, for 75% of the running period. In the first 5 days, mice ran at a velocity of 3.0 to 5.0 meters/minute that was increased to 16.5 to 18.0 meters/minute by day 10. From day 10 to day 28 mice ran at a velocity of 20.0 to 21.0 meters per minute. To control for any non-exercise effects of treadmill running (handling, novel environment, noise, and vibration) non-exercised groups were placed on the top of the treadmill apparatus for a time period equivalent to exercise training [Fuci T, 2000 #5581; Kodak G, 2001 #5583].

Brain tissue was collected at 4 days post-MPTP-lesioning prior to start of exercise paradigm, 7 days of exercise, and 28 days of exercise to examine the expression of TH and DAT striatal protein and expression of midbrain transcript mRNA, as well as striatal dopamine levels. Tissue for immunohistochemical analysis was fixed by transcardial perfusion and prepared as described (REF; Fisher, Jakowec). Tissues for western immunoblotting and *in situ* hybridization were harvested fresh following cervical dislocation and prepared as described (REF). Commercially available primary antibodies included rabbit polyclonal anti-TH (Chemicon, Inc, Temecula, CA), and mouse monoclonal anti-DAT (Chemicon, Inc, Temecula, CA). Antibody binding was visualized using an HRP-conjugated

secondary antibody using the ABC Elite kit (Vector Labs, Burlingame, CA) and development in DAB/H_2O_2. To ensure that differences in staining intensity were due to differences in antigen expression, multiple sections from each of the different treatment groups were handled in identical staining conditions concurrently. Control experiments excluding either primary antibody or secondary antibody were also carried out to verify staining specificity. For image analysis, 3 or 4 animals per treatment group and 10 to 12 sections per animal, were captured at low magnification, and digitized. The relative optical density (expressed as arbitrary units within the linear range of detection) of the dorsal lateral striatum was determined by subtracting the relative optical density of the corpus callosum as background.

Tissue for western blot analysis was dissected from the mid-striatum (section spanning Bregma 0.00 to 1.50) of at least 6 mice from each group and treated as previously described [Jakowec, 2004 #4800]. Immunoblots were exposed to primary antibody and then a fluorescent-conjugated secondary antibody and processed for image capture and analysis using the Licor Odyssey Imaging System (Lincoln, Nebraska).

Brains for *in situ* hybridization were quickly removed and frozen in isopentane on dry ice and tissues processed as previously described [Jakowec, 1995 #705; Jakowec, 2004 #4800]. Selected slides were dipped in NTB-2 (Kodak) photographic emulsion, developed in D-19 developer and counter stained with cresyl violet. To minimize potential sources of variation between different experiments, slides that were to be compared were processed in the same experiment using identical hybridization buffers, probe concentration, probe preparation, wash regimen, and emulsion exposure. Images of midbrain cells were captured using an Olympus BX-51 microscope and the computerized image analysis program Bioquant (Bioquant Imaging, Nashville, TN) was used to determine the number of emulsion grains above individual neurons within the substantia nigra pars compacta.

For statistical analysis, linear regression was performed to compare the rate of change in velocity of treadmill running between the two groups. Treatment groups were compared with a one-way ANOVA, followed by the Fisher post hoc test for comparison of multiple means. All analyses were performed with SPSS software (XX) or Instat (XX). Statistical significance was accepted at $P < 0.05$.

RESULTS

Effect of Treadmill Exercise on Motor Behavior

The treadmill exercise paradigm used in these studies is based on previous published studies, which showed that MPTP-lesioned + exercise mice had both running velocity and exercise duration below that of saline + exercise mice [Fisher, 2004 #6254]. By 21 days of exercise both groups had equal velocity and duration. Therefore, in these studies we carried out the treadmill exercise for 28 days, which corresponds to a time line where motor behavior benefits are observed. During the first 15 days of exercise the MPTP group typically ran at a velocity 1.5 to 2.0 meters/minute slower than the saline + exercise group. By day 11 both saline and MPTP exercise groups were run at velocities ranging from 15.0 to 18.5 meters/minute which increased to 20.0 to 22.0 meters/minute in the last 5 days of exercise. Both saline + exercise and MPTP + exercise groups were able to achieve the goal of 22.0 meters /minute for 60 minutes by exercise days 23 to 28. During the exercise period mice from all groups (exercise and non-exercise) were weighed weekly and there was no significant differences in body weight between the four groups.

HPLC Analysis of Striatal Dopamine and Its Turnover

HPLC analysis of striatal dopamine 4 days after MPTP-lesioning, which represented the first day of treadmill exercise, showed 96% depletion. Table 1 and Figure XX shows both the striatal dopamine

and turnover ratio from mice after either 7 or 28 days of treadmill exercise (representing 11 or 32 days post-MPTP-lesioning). At 7 days of exercise both the MPTP (22.2 ng dopamine / mg protein) and MPTP + exercise (2.8 ng dopamine / mg protein) mice were significantly different from the saline group (155.0 ng dopamine / mg protein) ($p < 0.05$). There was a slight suppression of dopamine in the MPTP + exercise group compared to the MPTP group but it was not statistically significant. After 28 days of exercise, both the MPTP (77.9 ng dopamine / mg protein) and MPTP + exercise (69.8 ng dopamine / mg protein) groups remained statistically different from the saline group ($p < 0.05$) but were not significantly different from each other. Interestingly, there was an elevation in striatal dopamine in the saline + exercise group (315.2 ng dopamine / mg protein) compared to the saline group (247.0 ng dopamine / mg protein) ($p < 0.05$). Analysis of turnover ratio defined as (DOPAC + HVA)/dopamine showed elevation in both the MPTP (2.26) and MPTP + exercise (1.31) groups at 7 days of exercise, which were both statistically different from saline group (0.20) ($p < 0.05$). The MPTP + exercise group was slightly reduced compared to the MPTP group. At 28 days of exercise the turnover ratios of the MPTP (0.36) and MPTP + exercise (0.34) groups were reduced to a level that was not significantly different from the saline group (0.26) or saline + exercise group (0.34).

Striatal Tyrosine Hydroxylase and Dopamine Transporter Protein Expression

Analysis of the relative expression of striatal TH and DAT protein using western immunoblotting were carried out in representative animals ($n = 4$) from all groups at either 7 or 28 days of treadmill exercise. These results are shown in Figure 2.

Put TH results here.

Analysis of the relative expression of DAT is Shown in Figure 2B. In the first week after MPTP-lesioning we observed an approximately 50% reduction in DAT-ir compared to saline mice. Following 7 days of exercise saline mice showed a slight elevation in the relative expression of DAT compared to saline non-exercised mice. This is in contrast to the MPTP-lesioned mice where the relative expression of DAT-ir is reduced in the MPTP + exercise mice compared to MPTP alone. When DAT-ir was analyzed after 28 days of exercise, there was no significant difference between the saline and saline + exercise groups. However, the suppression of DAT-ir was still observed in the MPTP + exercise group compared to the MPTP no exercise group.

Tyrosine Hydroxylase and Dopamine Transporter mRNA Expression

The relative expression of both TH and DAT mRNA transcripts in midbrain dopaminergic neurons was determined in all four groups after 28 days of exercise using in situ hybridization histochemistry in conjunction with grain counting in emulsion dipped sections. These data are shown in Figure 3A and 3B.

Put TH results here.

The highest level of DAT mRNA expression was observed in the saline group. Following the 28-day exercise regimen there was a 43% reduction in the number of grains in the saline + exercise group compared to the saline no-exercise group that was statistically significant ($p < 0.05$). The degree of DAT mRNA expression was also reduced to a similar level in both the MPTP and MPTP + exercise groups. There was no significant difference between the MPTP and MPTP + exercise groups.

DISCUSSION

The MPTP-lesioning regimen used in our studies involves a series of 4 injections of 20 mg/kg (free-base) leading to a 60 to 70% loss of nigrostriatal dopaminergic neurons and a 90 to 95% depletion of striatal dopamine [Jackson-Lewis V, 1995 #696](REF; Jakowec 2004). Intensive treadmill exercise was initiated 4 days after the last injection of MPTP when destruction of nigrostriatal dopaminergic neurons is complete. Therefore, these studies examined the effects of exercise on restoration of function of surviving neurons rather than neuroprotection.

Previous studies from Tillerson and colleagues have examined the effects of treadmill exercise in the MPTP-lesioned mouse model [Tillerson, 2003 #3613][Tillerson, 2002 #5650]. While our study employed young adult mice of 8 to 10 weeks of age using an acute MPTP-lesioning regimen of 4 injections of MPTP at 20 mg/kg 2 hours apart, their study used retired breeders (typically 5 to 8 months of age) and a mild degree of lesioning (15 mg/kg, 12 hours apart). Treadmill exercise was started 1 day before MPTP lesioning (and before cell death) while our exercise intervention was started 4 days after MPTP-lesioning when cell death is complete. This difference may indicate that both neuroprotection and neurorestoration are both involved if exercise intervention if introduced during the period of cell death while neurorestoration alone acts when exercise is introduced after cell death is complete. This may account for differences in the relative amount of striatal dopamine between these two studies. Our study showed no significant increase in striatal dopamine after 28 days of treadmill exercise between MPTP and MPTP + exercise mice while the Tillerson study showed an increase in striatal dopamine at 30 days of exercise in the MPTP-lesioned group (to 47.3% of saline + exercise representing a 4-fold increase compared to MPTP without exercise). Therefore, an important difference between these two studies is the potential degree of dopaminergic cell loss.

Immediate exposure to treadmill training within 12 hours of injury was associated with attenuation of dopamine loss. The investigators concluded that exercise might work largely through neuroprotective mechanisms since exercise was started within 12 hours of lesioning, and MPTP and 6-OHDA may take several days to complete cell death [Jackson-Lewis V, 1995 #696; Sauer, 1994 #1315].

In conclusion, exercise may be both neuroprotective and neurorestorative in the injured basal ganglia. It has been previously shown that initiating exercise at or during the time of neurotoxin-induced cell death is neuroprotective by attenuating striatal dopamine loss [Tillerson JL, 2003 #3613; Cohen AD, 2003 #5803]. Our previous studies have shown that a high intensity treadmill exercise paradigm initiated after the period of neurotoxin-induced cell death is neurorestorative as demonstrated through its beneficial effect on motor behavior. Alterations in both dopaminergic and glutamatergic neurotransmission in response to exercise may underlie the molecular mechanisms of this effect. The potential impact of this study is that exercise may not only play a role in prevention of Parkinson's disease but in restoring function in individuals who have been diagnosed with Parkinson's disease.

TABLE and FIGURE LEGENDS

Table 1: ***HPLC analysis of striatal dopamine and its metabolites.*** The concentration of dopamine, DOPAC, and HVA were analyzed in representative mice from all groups at either 7 or 28 days of exercise were analyzed. The turnover ratio is defined as [DOPAC + HVA]/Dopamine.

Figure 1: ***Analysis of striatal dopamine levels and dopamine turnover.*** The upper panel shows the HPLC analysis of striatal dopamine collected from mice in all 4 groups (saline, saline + exercise, MPTP, MPTP + exercise) at 3 days after MPTP-lesioning (1 day before start of exercise regimen), 7 days of exercise, and 28 days of exercise. The MPTP-lesioning regimen results in a XX% depletion of striatal dopamine. Note that after 28 days of treadmill exercise the levels of striatal dopamine in the MPTP and MPTP + exercise groups are not significantly different. The lower panel displays the turnover ration defined as [DOPAC + HVA]/DA for mice from all groups at times corresponding to the DA analysis in the upper panel. The turnover ration is elevated in the MPTP and MPTP + exercise groups at exercise day-7. Note that at exercise day-28 the turnover ratio is similar in both the MPTP and MPTP + exercise groups. For each time point and group 6 mice were used for HPLC analysis.

Figure 2: ***Analysis of Striatal Tyrosine Hydroxylase and Dopamine Transporter Protein Expression.*** The relative expression of striatal TH and DAT after MPTP-lesioning and between the different treatment groups was determined using immunohistochemistry and western immunoblotting.

Figure 3: ***Analysis of Tyrosine Hydroxylase and Dopamine Transporter mRNA in Midbrain Nigrostriatal Neurons.*** The relative expression of TH and DAT mRNA in midbrain dopaminergic neurons were determined using in situ hybridization histochemistry.